CHARACTERIZATION and MODIFICATION of ANTIOXIDANT PROTEINS from PLANT MATERIALS

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ABSTRACT

In this study, the radical scavenging and iron chelating capacity of proteins from heat treated (20 min at 90 ^{o}C) or thermally processed (20 min at 121 ^{o}C) chick-peas and kidney-beans were compared. Lyophilized crude protein extracts from chick-peas contained more protein (1.5-3 fold) and showed higher free radical scavenging (up to 2.3 fold) and iron binding capacity (up to 3 fold) than lyophilized crude protein extracts form kidney-beans. The thermal processing of chick-peas did not cause a significant change in the radical scavenging capacity of their lyophilized crude protein extracts, but improved the iron chelating capacity of these proteins almost 80 %. However, the thermal processing reduced both the radical scavenging and iron binding capacity of crude lyophilized proteins form kidney beans by 20-40 % and 60 %, respectively. Partial purification by ammonium sulfate precipitation or DEAE-cellulose chromatography increased the antioxidant capacity of thermally processed chick-pea proteins. The DEAE cellulose chromatography also showed the presence of 5 and 3 antioxidant protein fractions in heat treated and thermally processed chick-peas, respectively. Hot acidic hydrolysis at 80 ^{o}C for 30 min in presence of 1.5 M HCl increases the specific antioxidant activity of protein extracts, but causes the formation of undesired Maillard reaction products. Hot extraction at 85 ^{o}C for 30 min at pH 2.5 extracts the antioxidant proteins selectively, whereas 85 ^{o}C for 30 min at pH 9.5 extracts both antioxidant proteins and other proteins.

ÖZET

Bu çalışmada ısı uygulaması (90 ^{o}C'de 20 dak) veya ısıl işlem (121 ^{o}C'de 20 dak) uygulanmış nohut ve kuru fasulye proteinlerinin serbest radikalleri inhibe etme ve demir bağlama kapasitleri kıyaslanmıştır. Elde edilmiş sonuçlar, liyofilize edilmiş ham fasulye protein ekstraktlarına kıyasla, liyofilize edilmiş ham nohut protein ekstraktlarının protein içeriğinin 1.5-3 kat, antioksidant aktivitesinin 2.3 kat ve demir bağlama kapasitesinin 3 kat kadar daha yüksek olabileceğini göstermiştir. Isıl işlem uygulanması liyofilize ham nohut proteinlerinin serbest radikaller üzerindeki aktivitesini etkilememekte, ancak demir bağalma kapasitelerini yaklaşık % 80 artırmaktadır. Ancak, ısıl işlem uygulaması fasulyelerden elde edilen liyofilize ham protein ekstraktlarının serbest radikalleri inhibe etme ve demir bağlama kapasitesini sırasıyla % 20-40 ve % 60 oranında azaltmaktadır. Isıl işlem görmüş nohut ham protein ekstraktlarının amonyum sülfat veya DEAE-selüloz kolon kromatografisi ile kısmi olarak saflaştırılması onların serbest radikalleri inhibisyon kapasitesinde artışa neden olmuştur. DEAE-selüloz kromatografisi ayrıca, ısı uygulamış nohut protein ekstraktlarında 5, ısıl işlem uygulanmış nohut protein ekstraktlarında ise 3 antioksidant protein fraksiyonu bulunduğunu göstermektedir. Protein ekstraktlarının spesifik antioksidant aktivitesi 85 ^{o}C'de 30 dak 1.5 M HCl ile asidik hidrolizle artırılabilmekte, ancak bu işlem arzulanmayan Maillard reaksiyon ürünleri oluşturmaktadır. Diğer yandan pH 2.5 ve 85 ^{o}C'de 30 dak yürütülen ekstraksiyon selektif olarak antioksidant proteinlerin, pH 9.5 ve 85 ^{o}C'de 30 dak yürütülen ekstraksiyon ise antioksidant ve diğer proteinlerin ekstraksiyonu amacıyla kullanılabilmektedir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

The suspicious carcinogenic effects of synthetic antioxidants such as BHA and BHT on laboratory animals have raised significant concerns about the use of these chemical additives in foods (Madhavi et al. 1996c). Thus, recently, the demand of natural antioxidants has increased enormously (Madhavi et al. 1996c, Yang et al. 2000, Hwang et al. 2001). The natural compounds are not considered as chemicals and they are readily accepted by the consumers. Also, they have a GRASS (Generally Recognized As Safe) status and do not require toxicological testing. On the other hand, natural antioxidants are more expensive than the synthetic ones, since they generally need purification before used in food applications (Rajalakshmi and Narasimhan 1996). Also, most natural antioxidants effect food color and flavor adversely. For example, there are only several odorless and tasteless commercially available phenolic antioxidants and this limits the use of highly effective natural phenolic antioxidants in foods (Madhavi et al. 1996b, Reische et al. 1998). Ascorbic acid and its salts or derivatives are also important natural or natural identical antioxidants that have GRASS status. Due to their limited solubility in lipids, these antioxidants are not suitable for use in fat-containing food (Reische et al. 1998). However, after banning and limitations of using sulfites in fresh and processed fruits and vegetables, ascorbic acid and derivatives became the major sulfite alternatives to prevent enzymatic and non enzymatic browning in these products (Sapers et al. 1987, Yemenicioglu 2002). Other natural antioxidants include carotenoids which can be used in lipid systems as singlet oxygen quenchers if their yellow, orange, or red color is compatible with the food and tocopherols that have vitamin E activity in the diet (Reische et al. 1998). Proteins, protein hydrolysates, peptides, amines and amino acids are also an important group of natural antioxidants. The side chains of proteins show radical scavenging activity (Rajalakshmi and Narasimhan 1996) and chelating activity on metal atoms (Reische et al. 1998). Also, most proteins are tasteless and odorless, and due to their amphiphilic nature they can interact both with hydrophilic and hydrophobic food constituents sensitive to oxidation. Many proteins including casein, ovalbumin, oilseed proteins, gliadin, zein, bovine serum albumin, yam dioscorin, lactoferrin, sericin, carnosine, etc have been reported to

have an antioxidant activity (Rajalakshmi and Narasimhan 1996, Kouoh et al. 1999, Kim et al. 2001, Hou et al. 2001, Hu et al. 2003).

Legumes a large family of plants cultivated such as common bean (*Phaseolus vulgaris L.*), chickpea (*Cicer arietinum L.*), lentil (*Lens culinaris Medkus*), and soybeans (*Glycine max*) are good source of proteins. Due to their agricultural, economic, and nutritional values, legumes are consumed in the majority of Mediterranean countries (Lquari et al. 2002). However, the studies related to the antioxidant properties of legume proteins are concentrated mainly on soy proteins. Chen et al. (1995) have isolated six antioxidative peptides form protein hydrolyzates of β-Conglycinin which is the main soybean protein component. Chen et al. (1996 and 1998) have also studied the antioxidant activity of synthetic peptides designed based on an antioxidant peptide isolated from the soy protein hydrolizates. The antioxidant potential of soy protein hydrolizates in liposomal systems has been demonstrated by Pena-Ramos and Xiong (2002), whereas Hu et al. (2003) investigated the antioxidant activity of soy protein isolate in oil-in-water emulsions.

Dry beans are important source of proteins but have disadvantages such as low nutritional value due to limiting amounts of sulfur containing amino acids, low digestibility, low bioavailability of essential amino acids, presence of toxic and antinutritive factors, and absorption of nutrients by undefined non-protein substances (Friedman 1996). Chick-pea seeds, with their good balance of amino acids, high protein bioavailability, and relatively low levels of antinutritional factors may be potential ingredients for food products (Clemente et al. 1999). In the literature, there are limited studies related to the antioxidant activity of legume proteins other than the soy beans. The studies available include that of Okada and Okada (1998) who determined significant superoxide scavenging activity of water soluble proteins from broad beans and that of Wolosiak and Klepecka (2002) who reported that pea and bean albumins are much more effective in inhibiting the superoxide anion radical formation than the animal derived albumin preparations. There are no available studies related to the antioxidant activity of chick-pea proteins. Thus, in this study we have compared the antioxidant activity of crude or partially purified protein extracts from heat treated or thermally processed chick-peas and kidney-beans, isolated the major antioxidant protein fractions form chick-peas by anion exchange chromatography and tested the effects of different modification and extraction methods on antioxidant activity of chick-pea proteins.

CHAPTER 2

LIPID OXIDATION

2.1. Lipids

The term lipid refers to any naturally occurring non-polar substance that is nearly or totally insoluble in water but soluble in nonpolar solvents. The lipids are important bulk components in food and other biological systems and can be classified as (1) simple lipids (neutral acylglycerols and waxes), (2) compound lipids (phospholipids and glycolipids) and (3) derived lipids (carotenoids, lipid soluble vitamins, steroids etc.). The simple lipids are esters of glycerol and fatty acids (Neutral acylglycerols) or esters of long chain alcohols and long chain fatty acids (waxes). In compound lipids, in addition to alcohols and fatty acids, different compounds (phosphoric acid diesters and carbohydrates) exist in the structure. On the other hand, derived lipids are compounds that can not be neatly classified as simple or compound lipids. In fact, these are the fatty acids and alcohols which are the building blocks of simple and compound lipids (O'Keefe 1998). Unlike to carbohydrates and proteins, lipids possess only few reactive sites in the molecule. The major reactions of lipids involve the hydrolysis of their ester linkages and oxidation of their double bonds in fatty acids (Davidek et al. 1990). Lipid oxidation is one of the most frequent reactions causing the loss of quality in food products. It causes development of various off-flavors and off-odors in edible oils and fat-containing foods and this is called the oxidative rancidity (Nawar 1996). The lipid oxidation also decreases the nutritional quality of foods and forms some oxidation products that can be toxic (Madhavi et al. 1996a).

2.1.1. Basic Chemistry of Lipid Oxidation

The unsaturation (double bonds) of fatty acids makes them very sensitive to oxygen attack that cause complex chemical changes led to off-flavor formation in foods. This process is called autoxidation and it is characterized by three main stages (Erickson 1996, Jadhav et al. 1996); (1) initiation, (2) propagation and (3) termination.

2.1.1.1. Initiation

The initiation of autooxidation takes place by the abstraction of a hydrogen radical from the allylic methylene group of a fatty acid and formation of a lipid free radical (Davidek et al. 1990, Jadhav et al. 1996).

$\mathbf{RH \rightarrow R^{\cdot} + H^{\cdot}}$ (a) Formation of lipid free radical

A free radical is a highly reactive substance defined as a molecular entity having single unpaired electron (Kaur and Perkins 1991) and it may form by the effect of metal catalysts, irradiation or heat (Jadhav et al. 1996). Also, hydroperoxide decomposition may form free radicals. In fact, since the activation energy for the reaction of fatty acids with oxygen and production of free radicals ($RH + O_2 \rightarrow R^{\cdot}$) is high, it is proposed that the initiation reaction is started by the free radical formation by decomposition of hydroperoxides (Nawar 1996). Food materials always contain residual amounts of lipid hydroperoxides formed by the effect of singlet oxygen ($^1O_2$) or by oxidative enzymes such as lipoxygenase. By the effect of metal atoms and light, the hydroperoxides may decompose to free radicals such as alkoxy radical (b) and peroxy radical (c) and this may initiate oxidation.

$\mathbf{ROOH \rightarrow RO^{\cdot} + HO^{\cdot}}$ (b) Alkoxy radical formation from hydroperoxide

$\mathbf{2ROOH \rightarrow RO^{\cdot} + ROO^{\cdot} + H_2O}$ (c) Alkoxy and peroxy radical formation from hydroperoxide

(RH: lipid; R· : Lipid free radical; ROOH: Lipid hydroperoxide; ROO· : Lipid peroxy radical; RO· : Alkoxy radical; H· : Hydrogen radical, $^1O_2$: singlet oxygen; $^3O_3$: tripled oxygen)

2.1.1.2. Propagation

The free radicals are very reactive and they attack on molecular oxygen. Thus, in propagation step, free radicals are oxidized and converted into other free radical species

by chain reaction process (d). Thus, the initial formation of one free radical is responsible for the following chemical formation of the other radicals due to the chain reaction process.

$\mathbf{R^{\cdot} + {}^3O_2 \rightarrow ROO^{\cdot}}$ (d) Peroxy radical formation from lipid free radical

$\mathbf{ROO^{\cdot} + RH \rightarrow ROOH + R^{\cdot}}$ (e) Abstraction of a new H from another fatty acid

The newly produced lipid peroxy radicals ($ROO^{\cdot}$) start chain reactions with other molecules and form lipid hydroperoxides and new lipid free radicals (e). The free radical formed then again converted to peroxy radical and the peroxy radical abstract another H from another fatty acid. The peroxy radicals ($ROO^{\cdot}$) readily abstract hydrogen from the doubly allylic methylene groups. Such methylene groups are central CH_2 groups in the 'skipped diene' units (-CH=CH-CH_2-CH=CH-). Thus, in naturally occurring lipids containing linoleic or linolenic acid units, the doubly allylic methylene groups increase the oxidation potential (Jadhav et al. 1996). The reaction sequence in (d) and (e) may be repeated many times and a significant oxidative damage may occur only by the effect of small number of free radicals formed in the initiation step. The number of repeated reactions in the propagation step may depend on the concentration of the reactive lipid and free radicals in the reaction medium. The lipid hydroperoxides are the main autoxidation products and they are odorless and tasteless (Jadhav et al. 1996). However, they are relatively unstable and undergo numerous complex reactions such as substrate degradation and interaction. These reactions form many different undesirable compounds having various molecular weights and flavor thresholds (Nawar 1996).

So far lipid oxidation by chain propagation reaction based on abstraction of a hydrogen radical from the allylic methylene group of a fatty acid has been discussed. However, chain propagation reactions causing lipid oxidation may also occur by addition of free-radical to unsaturated bonds. Such chain propagation reactions, for example, occur in β-carotene that contains conjugated carbon-carbon double bonds (Jadhav et al. 1996).

2.1.1.3. Termination

Initially, the number of free radicals is very small. However, gradually their concentration increases and they start to interact with one another. In fact, since free radicals contain unpaired electron they tend to react each other to restore normal bonding (Kaur and Perkins 1991). The interaction of radicals and formation of non-radical compounds is termination. In reaction medium, the highly reactive free radicals are not stable and they are readily converted to other reactive species such as peroxy radical. This increases the concentration of peroxy radical in the medium and makes the combination of two peroxy radical (with the elimination of oxygen) the main termination reaction. The combination of peroxy radical with free radical is less frequent whereas combination of two free radical occur only at very low oxygen concentrations (Davidek et al. 1990).

$$R^{\cdot} + R^{\cdot} \rightarrow R\text{-}R$$

$$R^{\cdot} + ROO^{\cdot} \rightarrow ROOR \qquad \text{Termination reactions}$$

$$ROO^{\cdot} + ROO^{\cdot} \rightarrow ROOR + O_2$$

2.1.2. Effect of Lipid Oxidation on Food Systems and Human Health

Lipid oxidation is a chemical and biochemical reaction process that leads to the formation of free-radicals, hydroperoxides and many other products. The unstable hydroperoxides break down to aldehydes, ketones, hydrocarbons, acids and furans that form rancid off-flavors and off-odors in foods. Particularly, the cleavage products of hydroperoxides are responsible for the formation of rancid off-flavors (Davidek et al. 1990, Erickson 1998). The thermal degradation of lipid hydroperoxides also generates off-flavors, mainly as a result of the formation of carbonyl compounds (Jadhav et al. 1996). The reaction of lipid oxidation products with proteins also decreases protein solubility and nutritive value. The loss of nutritive value of proteins occurs mainly due to the destruction of tryptophan, oxidation of methyonine and binding lysine onto unavailable compounds (Davidek et al. 1990). The macromolecular substances produced by oxidized lipid-protein interactions are often dark colored. The oxidative reaction products can also cause discoloration by reacting with other food pigments

(Davidek et al. 1990) and they reduce the availability of vitamins such as A, D, E, C and folate (Jadhav et al. 1996).

Lipid oxidation and it's radical products have different effects on human health. Some of the diseases related to oxidative reactions in body are coronary heart disease (e.g. heart attack), aging, DNA damage, parkinsonism, carcinogenesis and tumour promotion (Jadhav et al. 1996).

CHAPTER 3

ANTIOXIDANTS

3.1. Definition of Antioxidant

Antioxidants are group of chemicals that protect biological systems against the potential harmful effects of processes, or reactions that cause oxidation (Decker 1998). The U.S Food and Drug Administration defines antioxidants as "preservatives that specifically retard deterioration, rancidity, or discoloration due to oxidation" (Specchio 1992). In most raw materials, the antioxidants exist as natural components. However, during food manufacturing and storage the natural antioxidants are exhausted. Thus, the addition of antioxidants to food products is necessary to keep food quality and extend shelf-life.

Antioxidants to be used in food products should have some characteristic properties. For example, they should be inexpensive, nontoxic, effective even at low concentrations, stable, and have no or minimal effect on color, flavor, and odor properties of food products (Reische 1998, Rajalakshmi and Narasimhan 1996). The use of antioxidants in food products is regulated by laws and international standards which are determined by international associations such as Joint FAO/WHO Expert Committee on Food Additives (JECFA), and the European Community's Scientific Committee for Food (SCF) (Rajalakshmi and Narasimhan 1996).

3.2. Classification of Antioxidants

Antioxidants have different activity mechanisms such as free radical scavenging, inactivation of peroxides and other reactive oxygen species, chelation of metals, and quenching of secondary lipid oxidation products (Decker 1998). According to their mechanism of action antioxidants are classified as primary antioxidants and secondary antioxidants (Rajalakshmi and Narasimhan 1996).

3.2.1. Primary Antioxidants

Primary antioxidants donate hydrogen or electrons to lipid free-radicals and interrupt radical chain reactions by converting them into more stable nonradical products (a) (Rajalakshmi and Narasimhan 1996). By the same mechanism they also react with lipid peroxy and alkoxy radicals and nonlipid free radicals (b,c). In fact, the primary antioxidants are most effective before the initiation step which is called induction period, where the antioxidants are consumed and free-radicals are formed (Reische, 1998). Besides H donation, the primary antioxidants also interact with lipid free radicals and form lipid-antioxidant complexes (Rajalakshmi and Narasimhan 1996) or they can reduce hydropeoxides to hydroxy compounds (Reische 1998).

$AH + R^{\cdot} \rightarrow A^{\cdot} + RH$	(a) H donation of antioxidant to lipid free radical
$AH + ROO^{\cdot} \rightarrow A^{\cdot} + ROOH$	(b) H donation of antioxidant to lipid peroxy radical
$AH + RO^{\cdot} \rightarrow A^{\cdot} + ROH$	(c) H donation of antioxidant to lipid alcoxy radical

Following donation of H from antioxidants, the antioxidant radicals formed further interfere with the chain-propagation reactions by inhibiting the peroxy or alcoxy lipid radicals (d,e). The antioxidant radicals also react with each other and contribute to termination reactions (f).

$ROO^{\cdot} + A^{\cdot} \rightarrow ROOA$	(d) Reaction of peroxy radical with antioxidant radical
$RO^{\cdot} + A^{\cdot} \rightarrow ROA$	(e) Reaction of alcoxy radical with antioxidant radical
$A^{\cdot} + A^{\cdot} \rightarrow AA$	(f) Reaction of antioxidant radicals

Primary antioxidants show their activity even at very low concentrations. However, at very high concentrations they may act as prooxidants. The synthetic phenolic antioxidants are the major primary antioxidants (Rajalakshmi and Narasimhan 1996, Reische 1998). However, although these antioxidants are highly effective to prevent autooxidation, only a few of them is approved for food applications. The major considerations of acceptability of synthetic phenolic antioxidants are potential toxicity and/or carcinogenicity of these compounds. The examples of these synthetic antioxidants include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT),

propyl gallate (PG), and tertiary butylhydroquinone (TBHQ). The natural phenolic antioxidants and tocopherols can also act as primary antioxidants.

3.2.2. Secondary Antioxidants

The secondary or preventive antioxidants may act through different mechanisms to slow down the rate of oxidation. These antioxidants are also called synergists, since they promote the antioxidant activity of primary antioxidants. However, they do not convert free radicals to more stable products (Reische 1998). The main types of secondary antioxidants include oxygen scavengers and reducing agents, chelators and singlet oxygen quenchers. Also, there are some secondary antioxidants that function by decomposing lipid peroxides into stable end products (Rajalakshmi and Narasimhan 1996).

Oxygen scavengers and reducing agents act by scavenging oxygen and donating H atoms to peroxy radicals and primary antioxidants. The H donation to primary antioxidant radicals regenerates primary antioxidants and this enables using primary antioxidants more effectively (Rajalakshmi and Narasimhan 1996). Example antioxidants in this group include ascorbic acid and its derivatives and sulfides.

The chelators, on the other hand, include ethylenediaminetetraacetic acid (EDTA), citric acid, tartaric acid, citrate esters, phytic acid, lecithin and polyphosphates (Rajalakshmi and Narasimhan 1996, Reische 1998). These substances form complexes with prooxidant metals such as iron and copper and increase the effect of oxygen scavengers and primary antioxidants significantly. The metals accelerate oxidation reactions by acting as catalysts in free radical formation reactions. They can also lower the activation energy of initiation step. To form active radical species metal atoms can either interact directly with lipids (g) or with hydroperoxides (h,i). These reactions can be periodic with regeneration of the lower oxidation state of the metals (Reische 1998).

$$\mathbf{RH + M^{(n-1)} \rightarrow M^{n+} + H^{+} + ROO}$$ (g) Metal atom -lipid interaction

$$\mathbf{ROOH + M^{(n+1)+} \rightarrow Mn^{+} + H^{+} + ROO}$$ (h) Metal atom (lower oxidation state)-hydroperoxide interaction

$$ROOH + Mn^{+} \rightarrow M^{(n+1)+} + OH^{-} + RO$$ (i) Metal atom (higher oxidation state)-hydroperoxide interaction

The metals in their lower oxidation states accelerate hydroperoxide degradation more than metals in their higher oxidation states (Reische 1998). Thus, in presence of metals, reducing agents such as ascorbic acid act as prooxidants by converting metals such as Fe^{+3} and Cu^{+2} to their lower oxidation states (Fe^{+2} and Cu^{+}) (Madhavi et al. 1996b).

Singlet oxygen quenchers, on the other hand, are secondary antioxidants that deplete high energy of singlet oxygen and dissipate the energy in the form of heat (Reische 1998). Singlet oxygen is a high energy molecule that is responsible for the photooxidation of unsaturated fats and the subsequent generation of hydroperoxides (Nawar 1996, Reische 1998).

3.2.3. Miscellaneous antioxidants

Miscellaneous antioxidants are compounds that act as primary antioxidants or secondary antioxidants. The natural phenolic compounds such as flavonoids and related compounds, proteins, amino acids, Maillard reaction products, nitrites and nitrates, carotenoids, zinc, glucose oxides, superoxide dismutase and catalase and glutathione peroxidase enzymes are some examples for miscellaneous antioxidants (Rajalakshmi and Narasimhan 1996). With their chain breaking properties the natural phenolic compounds can act as primary antioxidants. Carotenoids such as β-carotene, lycopene and lutein are singlet oxygen quenchers. Proteins and Maillard reaction products act as chelator and radical scavenger (Lindsay 1996, Jadhav et al. 1996). Enzyme glucose oxidase is an oxygen scavenger (Labuza and Breene 1989), superoxide dismutase-catalase enzyme mechanism degrades reactive oxygen species to water and oxygen, glutathione peroxidase reduces peroxides to alcohols (Nordberg and Arner 2001). Zinc strongly inhibits lipid peroxidation at the membrane level by preventing iron binding (Rajalakshmi and Narasimhan 1996).

3.3. Sources of antioxidants

3.3.1. Synthetic antioxidants

The antioxidants used in foods are mostly synthetic antioxidants such as BHT, BHA, PG and TBHQ (Rajalakshmi and Narasimhan 1996). From these antioxidants, the synthetic phenolic antioxidants, BHT and BHA, has a particular importance, since they are the most preferred food antioxidants. These antioxidants are strongly lipophilic and used extensively in oil-in-water emulsions. They are also fairly thermostable antioxidants which are suitable for thermally processed food (Reische 1998). Moreover, BHA and BHT are steam volatile. Thus, they easily diffuse into food lipid layers and inhibit oxidation when incorporated into food packaging materials (Madhavi and Salunkhe 1996). On the other hand, TBHQ is a very thermostable antioxidant which is very suitable for frying applications. It is more effective in vegetable oils than BHA and BHT and shows a good synergism with sitric acid (Reische 1998). The other synthetic antioxidant is PG which is not suitable for frying applications due to its less thermostable nature. Since PG forms undesirable dark colored complexes with iron and copper, its preparations should be combined with chelators (Reische 1998).

3.3.2. Natural antioxidants

Recently, significant concerns have been raised related to the use of synthetic antioxidants in foods (Madhavi et al. 1996c). Particularly, the suspicious carcinogenic effects of BHA and BHT on laboratory animals increased the demand of natural antioxidants enormously (Madhavi et al. 1996c, Yang et al. 2000, Hwang et al. 2001). Since they are not considered as chemicals, natural antioxidants are readily accepted by the consumers. Also, the natural antioxidants have a GRASS (Generally Recognized As Safe) status and do not require toxicological testing. However, because of their lower effectiveness than synthetic antioxidants, natural antioxidants need mostly a purification before used in food applications (Rajalakshmi and Narasimhan 1996). This makes natural antioxidants more expensive than the synthetic ones and increases the costs. Also, most natural antioxidants effect food color and flavor adversely. In fact, this is one of the greatest handicaps for the use of highly effective natural phenolic

antioxidants in foods. For example, their strong flavor is the main limitation for the use of herb and spice extracts rich in phenolic acids and flavonoids and tea extracts rich in catechins (Reische et al. 1998). On the other hand, the rosemary extract containing diterpene phenolics, carnisol and carnosic acid, is one of the few commercially available odorless and tasteless phenolic extracts (Medhavi and Salunkhe 1996, Reische et al. 1998).

Ascorbic acid and its salts such as sodium or calcium ascorbate or derivatives such as erythorbic acid and ascorbyl palmitate are other important natural or natural identical antioxidants that have GRASS status. Due to their limited solubility in lipids, except the more lipid soluble ascorbyl palmitate, they are not suitable for use in fat-containing food (Reische et al. 1998). However, after banning and limitations of using sulfites in fresh and processed fruits and vegetables, ascorbic acid and derivatives became the major sulfite alternatives to prevent enzymatic and non enzymatic browning in these products (Sapers and Ziolkovski 1987, Yemenicioglu 2002).

Carotenoids such as β-carotene, licopene, isozeaxanthin, lycopen and lutein are also natural lipid soluble antioxidants that are used as singlet oxygen quenchers. However, these natural antioxidants can be used only when their yellow, orange, or red color is compatible with the food. Another lipid soluble natural antioxidant group is tocopherols that have vitamin E activity in the diet (Reische et al. 1998). These natural antioxidants present in relatively large amounts in most oil seeds and pass into crude oil during pressing and extraction (Davidek et al. 1990). However, the tocopherols show more antioxidant activity in lard and animal fats than in edible oils (Davidek et al. 1990).

Proteins, protein hydrolysates, peptides, amines and amino acids are also an important group of natural antioxidants. The antioxidant groups in proteins may show radical scavenging activity (Rajalakshmi and Narasimhan 1996). Also, some iron binding proteins can act as chelating agents (Reische et al 1998). Detailed information about the antioxidant activity of proteins has been given in Chapter 4.

CHAPTER 4

PROTEINS AND THEIR FUNCTIONAL PROPERTIES

4.1. Amino Acids

Amino acids are the building blocks of proteins. They consist of a hydrogen atom, an amino group, a carboxyl group and a side chain R group covalently attach to an α-carbon atom (Figure 4.1). Depending on the position of α-amino group the amino acids are designated D (dextro from latin dexter, right) and L (levo from latin leaves, left).

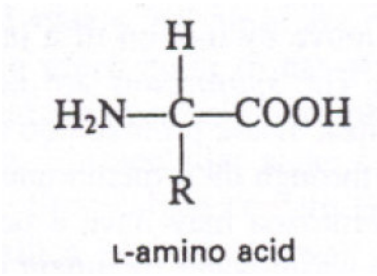


Figure 4.1. Amino acid structure (Shuler and Kargi 2002)

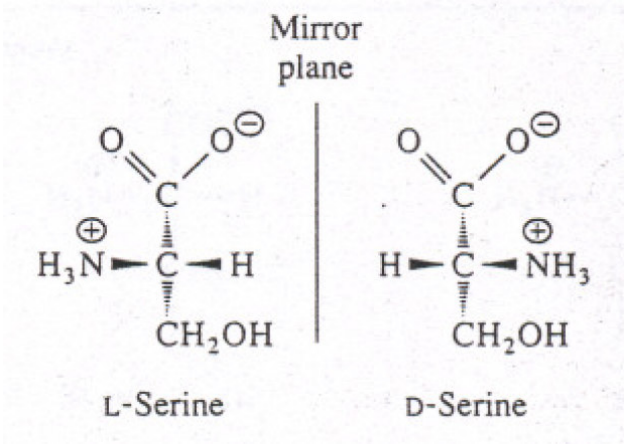


Figure 4.2. Stereoisomers of chiral amino acids (Horton et al. 1996a)

Natural proteins are composed of 20 amino acids linked end to end through peptide bonds. Despite the limited number of amino acids, variations in the amino acid

sequence give limitless number of proteins. With the exception of glycine, having H atom as R, the 19 amino acids contain at least a single chiral or asymmetric α-carbon atom. Thus, minimum two stereoisomers that have nonsuperimposible mirror images can exist for each of 19 aminoacid (Fig. 4.2). Such stereoisomers are called enantiomers. Two of the 19 amino acid contains two chiral carbon atoms each and therefore have four possible stereoisomers each (Voet and Voet 1995a, Horton et al. 1996a)

Chemical and physical properties of amino acids such as net charge, solubility, chemical reactivity, and hydrogen bonding potential depend on the type of side chain groups.

4.1.1. Properties of Amino Acid Side Chains

Side chains contain only five different atoms (H, C, N, O, and S). Some side chains are nonpolar and thus hydrophobic whereas others are polar or ionizable at neutral pH and therefore hydrophilic. The number of hydrophilic and hydrophobic amino acids is the main factor that dictates the shape of folded protein in water. According to the chemical properties of their side chains amino acid may be classified as aliphatic, aromatic, sulfur-containing, alcohols, bases, acids and amides (Mathews and Van Holde 1996, Horton et al. 1996a).

4.1.1.1. Aliphatic R Groups

Alanine (Ala, A), valine (Val, V), leucine (Leu, L), and isoleucine (Ile, I) have aliphatic side chains that made up entirely of highly hydrophobic methylene groups. Although these amino acids have no reactive functional groups they play an important role in the conformation of the proteins by their tendency to hide away from water (Stryer 1996, Horton et al. 1996a). The other member of this group Glysine (Gly, G), on the other hand, is the simplest amino acid with a H in its R group. Glycine plays a unique role in the confirmations of proteins since it is small enough to fit into small hollows in protein structure.

4.1.1.2. Aromatic R Groups

Phenylalanine (Phe, F), tyrosine (Tyr, Y), and tryptophan (Trp, W) have nonpolar hydrophobic aromatic rings in their side chains. Benzene ring on the side chain of phenylalanine makes it more hydrophobic than tyrosine and tryptophan. Tyrosine and phenylalanine are structurally similar. In tyrosine, the para-hydrogen of phenyl alanine is replaced with a hydroxyl group and this makes tyrosine a phenol. In tyriptophan, on the other hand, a bicyclic indole group replaces the benzene ring.

Aromatic R groups absorb UV light. For example tyrosine and tryptophan absorb UV light at 280 nm, whereas phenylalanine absorbs UV light weakly at 260 nm. Since most proteins have these amino acids in their structure, absorbance at 280 nm is frequently used to estimate protein concentration.

4.1.1.3. Sulfur-containing R Groups

Methionine (Met, M), and cysteine (Cys,C) have sulfur-containing R groups. Methionine is very hydrophobic due to nonpolar methyl thioeter group in its side chain. Although it is hydrophobic cysteine side chain (sulfhydryl group, -SH) is very reactive. This group is polarizable and it has an ability to form weak hydrogen bonds with oxygen and nitrogen. Moreover cysteine side chain becomes negatively charged by ionization.

The oxidation of cysteine causes the formation of a disulfide called cystine. Cystine contains two oxidized cysteine molecules linked by disulfite bonds. Oxidation of sulfhydryl groups of cysteine molecules occur more readily at alkaline pH values which these groups exist ionized. The disulfide bonds, by cross-linking cysteine residues in peptide chains, play an important role to stabilize three-dimensional structures of proteins (Branden and Tooze 1998, Ludescher 1996, Horton et al. 1996a).

4.1.1.4. Side Chains with Alcohol Groups

Uncharged polar side chains of the serine (Ser, S), and threonine (Thr, T) contain β-hydroxyl groups that give hydrophilic character to these side chains. However, unlike to the more acidic phenol side chain of tyrosine, these polar groups do

not undergo ionization (protonation-deprotonation) reactions in the pH range of 1 to 14. However, these groups can react within active sites of enzymes. Also, both serine and threonine side chains are suitable for phosphorylation or fatty acid esterification of proteins, since their hydroxyl group can react with acids to form esters (Ludescher 1996)

4.1.1.5. Basic R Groups

Histidine (His, H), lysine (Lys, L) and arginine (Arg, R) are nitrogenous bases that contain hydrophilic R groups positively charged at pH 7. Thus, these amino acids give the positive charges of proteins. Especially the most basic arginine is the main amino acid responsible from the positive charges of proteins.

4.1.1.6. Acidic R Groups and Amides

Aspartate (Asp, D) and glutamate (Glu, E) are dicarboxylic amino acids and are negatively charged at pH 7. In addition to their α-carboxyl groups, aspartate possesses a β-carboxyl group, and glutamate possesses a γ-carboxyl group. Because the side chains of aspartate and glutamate are ionized at pH 7, they give negative charges on proteins. Aspartate and glutamate are sometimes called aspartic acid and glutamic acid.

Asparagine (Asn, N) and glutamine (Gln, Q) are the amides of aspartic acid and glutamic acid, respectively. Although the side chains of asparagine and glutamine are uncharged, these amino acids are highly polar and are often found on the surface of proteins where they can interact with water molecules. The polar amide groups of asparagine and glutamine can also form hydrogen bonds with atoms in the side chains of other polar amino acids.

4.1.2. Hydrophobicity of Amino Acid Side Chains

Since the hydrophobic interactions are the main driving forces affecting protein folding, the amino acids' degree of hydrophobicity gives some important details about the major amino acids having roles in protein folding. The various side chains of amino acids range from highly hydrophobic to highly hydrophilic. The relative hydrophobicity

or hydrophilicity of each amino acid is called its hydropathy. Hydropathy value is calculated from the free energy change for transfer of an amino acid residue from the interior of a lipid bilayer to water. Amino acids with highly positive hydropathy values are considered hydrophobic whereas those with the largest negative values are hydrophilic. Table 4.1 shows the hydropathy scale for amino acid residues. This scale has been used to predict which segments of membrane proteins are likely to be embedded in the lipid bilayer.

Table 4.1. Hydropathy scale for amino acids (Horton et al. 1996a)

Amino acid	**Fee-energy change for transfer (kJ/mol)**
Highly hydrophobic	
Isoleusine	3,1
Phenylalanine	2,5
Valine	2,3
Leucine	2,2
Methionine	1,1
Less hydrophobic	
Tryptophan	1,5
Alanine	1,0
Glycine	0,67
Ctsteine	0,17
Tyrosine	0,08
Proline	-0,29
Threonine	-0,75
Serine	-1,1
Highly hydrophilic	
Histidine	-1,7
Glutamate	-2,6
Asparagine	-2,7
Glutamine	-2,9
Aspartate	-3,0
Lysine	-4,6
Arginine	-7,5

4.1.3. Ionization of Amino Acids

The physical properties of amino acids are influenced by the ionic states of the α-carboxyl and α-amino groups and possible ionizable groups in their side chains. Depending on the pH of medium, the α-carboxyl and α-amino groups may present at different ionic states (Horton et al. 1996a). At pH values approaching neutrality amino acids exist as dipolar ions (Zwitterions). The pH at which the dipolar ion is electrically neutral is called the ioelectric point (pI). Also, amino acids may behave as an acid or as a base (Fig. 4.3). In other words, amino acids are amphoteric.

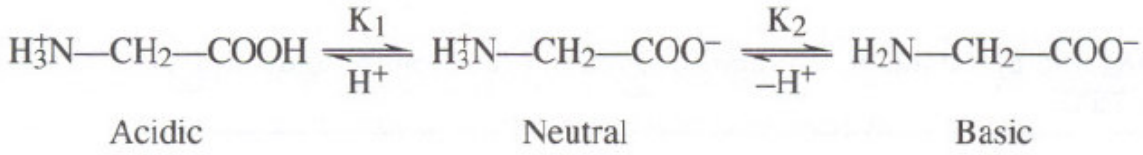


Figure 4.3. The ionic states of amino acids in different mediums (Damodaran 1996a)

As indicated above, in all amino acids, the α-carboxyl and α-amino groups can be ionized but in 7 of the 20 amino acids the side chain (R group) is also ionizable (Horton 1996a, Ludescher 1996) The amino acids which have ionizable side chain are cysteine, tyrosine, aspartic acid, glutamic acid, lysine, arginine, and histidine. The ionic states of amino acid side chains influence the three-dimensional structures and biological functions of proteins. In addition, a number of ionizable amino acid residues are involved in catalysis by enzymes. Thus, better understanding of the ionic properties of amino acids helps better understanding of enzyme mechanisms.

4.2. Proteins

Proteins are macromolecules that contain covalently linked and folded chains of amino acids and their biological function depends completely on their conformation (Horton et al. 1996b). The proteins have different kinds of structural organization such as primary, secondary, tertiary and quaternary structures.

4.2.1. Primary Structure

The primary structure of a protein refers to the linear sequence in which the constituent amino acids are covalently linked end to end through peptide bonds (also known as amide bond). The peptide linkage results from condensation of the α-carboxyl and α-amino group of two amino acids with removal of a water molecule (Fig 4.4).

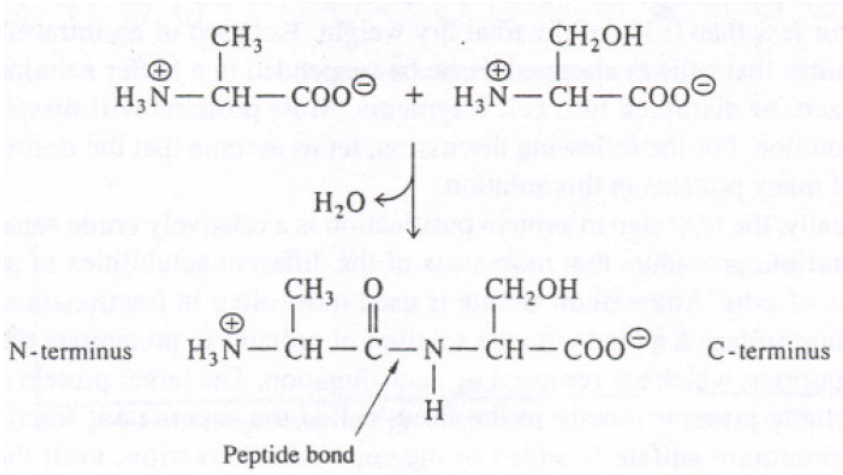


Figure 4.4. Peptide bond formation (Horton et al. 1996a)

The peptide chains are named according to the number of amino acids they contain. Dipeptides contain two, tripeptides contain three, oligopeptides contain several up to about 20 amino acids. Polypeptide refers to chains of usually more than 20 amino acids (Horton et al. 1996a) In a polypeptide, the free amino group at the end of the peptide chain is called N-terminus (amino terminus) whereas at the opposite end there is a free carboxyl group named C-terminus. The N and C terminus carries positive and negative charges at neutral pH, respectively. The side chains of the constituent amino acids also contribute to the net charge of protein molecules (Horton et al. 1996a).

The primary structure defines only the linear sequence of the amino acid residues but it gives no information about conformation of the protein. The primary structure of a protein determines its physicochemical, structural, biological properties and functions and it is like a code for formation of conformation (Damodaran 1996a).

4.2.2. Secondary Structure

The secondary structure refers to regular arrangements of polypeptides in terms of different secondary structures such as helical forms, β-structures and random coil. The helical structures are formed by rotation of bonds around the N-Cα and Cα-C^1 atoms (Fig. 4.x). The angles of rotation around the N-Cα and Cα-C^1 atoms are called the phi (Φ) and the psi (ψ), respectively. Depending on these angles different types of helical structures may be formed such as α-helix (Fig. 4.5), π-helix and 3_{10}-helix. However, the most frequently observed helical structure in proteins is α-helix structure that have a phi of (-58, +58) and the psi of (-47,+47) (Voet and Voet 1995b, Horton et al. 1996b) The helical structures are stabilized by H bonds formed between the –C=O of each peptide bond and the –NH of the peptide bond four amino acid residues away (Fig. 4.6). However, this is one of the most labile structures in proteins and may easily be disturbed by the effect of different physical factors such as heating and pressure.

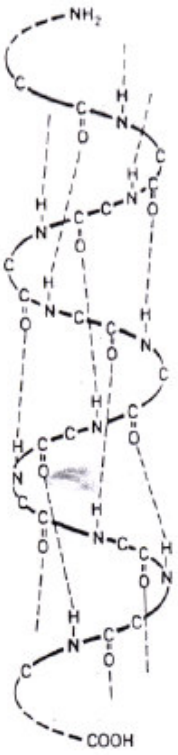


Figure 4.5. The structure of α-helix (Segel 1968)

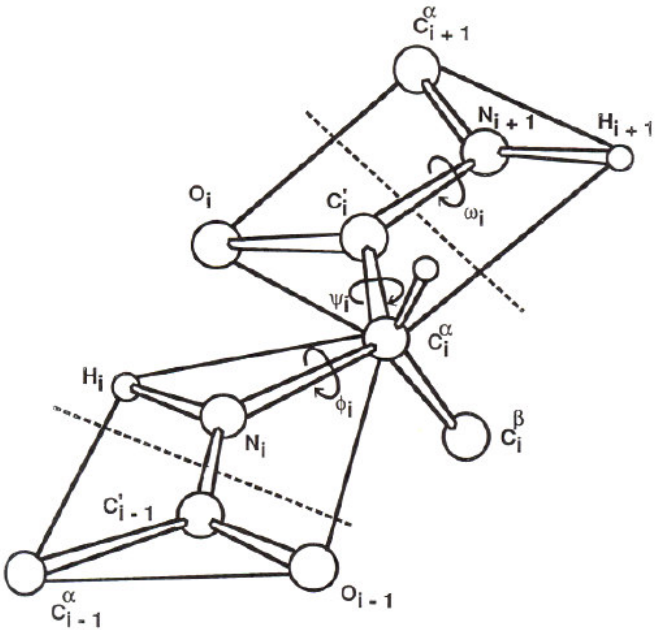


Figure 4.6. The configuration of the atoms of peptide units (Damodaran 1996a)

Another secondary structure is β-structures. These are zig-zag structures, more stretched than the helical forms. The formation of these structures occurs by suppression of H bonds in helical structures simply by heat. Each extended segment contains usually 5-15 amino acid residues and it is called β-stand. Different β-stands may be aligned and form H bonds to form β-sheets. The H bonds in the β-sheets are formed only between segments but not within segments as observed in helical structures. The β-stands may interact in two ways; (1) the amino acids in the aligned β-stands can all run in the same biochemical direction (parallel), N terminal to C terminal, or (2) amino acids in successive strands can have different directions, N terminal to C terminal of one stand follow the C terminal to N terminal of the other (Fig. 4.7).

The stability of antiparallel β-sheets are much more than the stability of the parallel β-sheets and both forms are more stable than the helical structures (Horton et al. 1996b, Damodaran 1996a)

(a) (b)

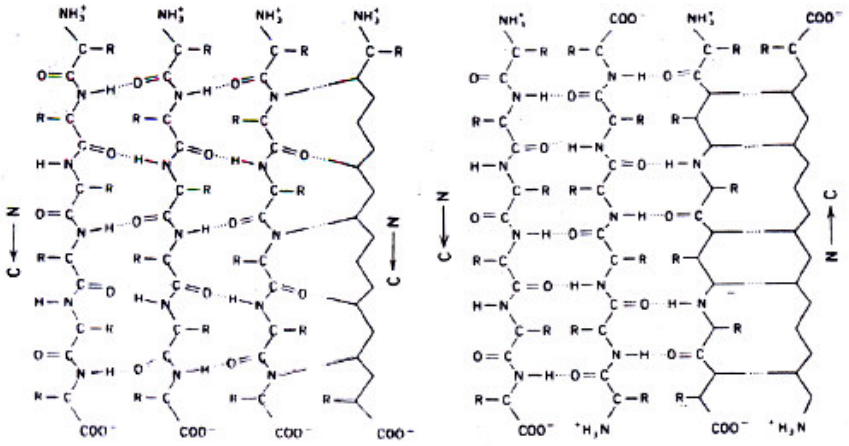


Figure 4.7. The parallel (a) and antiparalle (b) β-sheets (Segel 1968)

4.2.3. Tertiary and Quaternary Structures

Tertiary structure refers to the three dimensional organization of a protein with secondary structure segments such as helical structures, β-structures and random coil. The driving force for the formation of tertiary structures is hydrophobic interactions that cause the folding of polypeptide due to coalescence and burial of the hydrophobic amino acid residues through the internal parts. In tertiary structure that contains only a single polypeptide chain connection of the secondary structure elements is conducted by the loop regions. There are many different loop regions that connect secondary structure elements. For example, hairpin loop connects two adjacent antiparallel β-stands, alpha-alpha loops connect to helical structures, beta-alpha-beta loops (or motifs) connect two parallel β-strands, greek-key motif links four or more antiparallel β-structures (Horton et al. 1996b). In tertiary structure the hydrophilic helical structures exist at the surface of protein whereas the hydrophobic β-structures exist at the center (Fig 4.8). If the number of hydrophobic amino acids in the polypeptide is very high, the protein folds to form a compact and globular shape. In contrast, too much hydrophilic amino acids in the structure dictate the formation of a rod like extended conformation. The tertiary structures are stabilized by different types of interactions such as H bonds, ionic interactions, dipole-dipole interactions, hydrophobic forces and covalent disulfide bonds (Segel 1968).

The quaternary structure refers to the three dimensional organization of a protein when it contains more than one polypeptide. The driving farce for the formation of

protein quaternary structures is again hydrophobic interactions. Proteins that contain more than 30 % hydrophobic amino acid residues tend to form quaternary structures. In such proteins it is physically not possible to bury all the hydrophobic amino acids to the internal parts of protein. Thus, two or more proteins having hydrophobic patches exposed at the surface tend to come together about their hydrophobic surfaces. This mechanism may cause the formation of protein dimers, trimers or tetramers etc (Fig 4.9) (Damodaran 1996a).

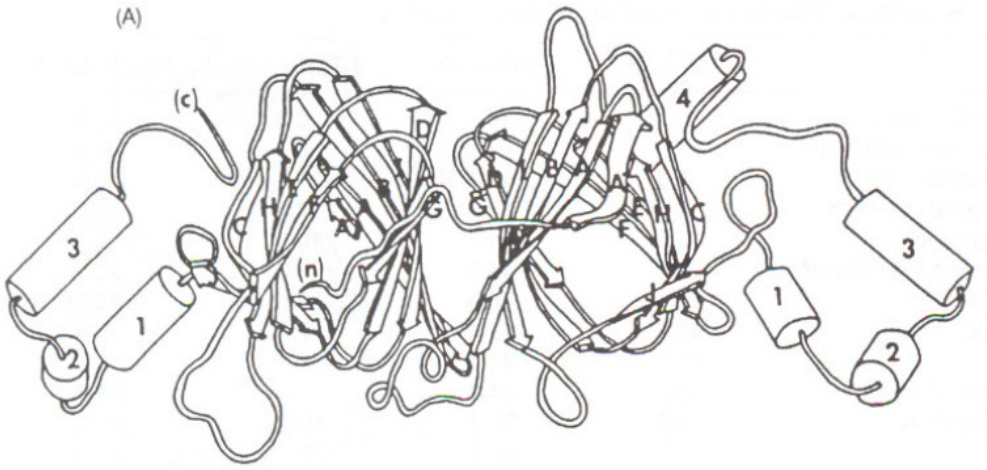


Figure 4.8. Tertiary structure of phaseolin subunit (Damodaran 1996a).

The specific protein-protein interactions such as hydrogen bonding, hydrophobic interactions, and electrostatic interactions, on the other hand, are the main interactions that stabilize the quaternary structures.

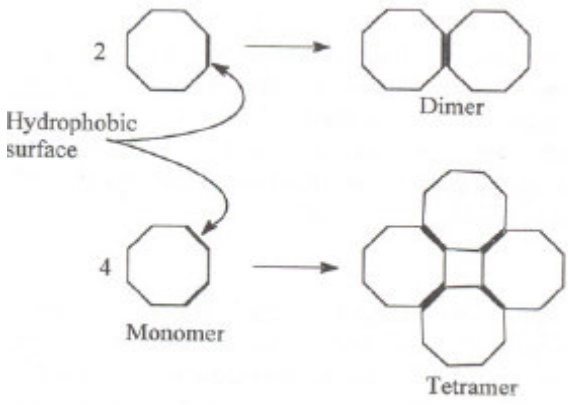


Figure 4.9. Schematic representation of the formation of quaternary structures (Damodaran 1996a)

4.3. Functional Properties of Proteins

Protein functionality is defined as physical and chemical properties which affect the behavior of proteins in food system during processing, storage, preparation and consumption (Damodaran 1996a). The physical and chemical properties affecting the functions of protein depend on their size, shape, amino acid composition and sequence, net charge and distribution of charges, hydrophobicity/hydrophilicity ratio, secondary, tertiary and quaternary structures, molecular flexibility/rigidity and ability to interact with other components (Damodaran 1996a). The functional properties of proteins can roughly be classified as; (1) hydrodynamic properties which include viscosity (thickness), gelation and texturization and (2) protein surface–related properties which include wettability, dispersability, solubility, foaming, emulsification and fat and flavor binding. Some proteins posses only one of these functions whereas some other proteins are multifunctional. For example, proteins of animal origin such as milk, egg and meat proteins are mostly capable of performing multiple functions. Especially, egg white proteins show multiple functions such as gelation, emulsification, foaming, water binding, and heat coagulation (Damodaran 1996a). Different functions of proteins in foods are given in Table 4.2.

Table 4.2. Functional roles of food proteins (Damodaran 1996b)

Function	Mechanism	Food System	Protein Source
Solubility	Hydrophilicity	Beverages	Whey proteins
Viscosity	Water binding, hydrodynamic size, shape	Soups, gravies, salad dressings	
Water binding	H-bonding, ion hydration	Meat sausages, cakes, breads	Muscle proteins, egg proteins
Gelation	Water entrapment and immobilization, network formation	Meats, gels, cakes, baked goods, cheeses	Muscle proteins, egg and milk proteins
Cohesion adhesion	Hydrophobic, ionic, and H-bonding	Meats, sausages, pasta, baked goods	Muscle proteins, egg proteins, whey proteins
Elasticity	Hydrophobic bonding, disulfide cross-links	Meats, baked goods	Muscle proteins
Emulsification	Adsorption at interfaces, film formation	Sausages, bologna, soups, cakes, dressings	Muscle proteins, egg proteins, milk proteins
Foaming	Interfacial adsorption, film formation	Whipped toppings, ice cream, cakes, desserts	Egg proteins, milk protein
Fat and flavor binding	Hydrophobic bonding, entrapment	Simulated meats, baked goods, doughnuts	Milk proteins, egg proteins

4.3.1. Protein Hydration

One of the essential components of food is water which effects the rheological and textural properties of foods depending on its interaction with other food components such as proteins and polysaccharides. The interaction of water with proteins may effect the functional properties of the proteins such as dispersibility, wettability, swelling, solubility, thickening/viscosity, water-holding capacity, gelation, coagulation, emulsification and foaming capacity. Thus, to better understand the possible functions of proteins in food, it is essential to analyze the hydration of proteins.

Water molecules bind to proteins through their charged groups (ion-dipole interactions); backbone peptides groups: the amide groups of asparagine and glutamine; hydroxyl groups of serine, threonine, tyrosine residues (dipole-dipole interactions); non-polar residues (dipole-induced dipole interaction, hydrophobic hydration). When a dry protein interacts with water, the initial hydration occurs at the sites of ionizable groups of protein. Then, water clusters form near the polar and charged protein surfaces and hydration at the polar surfaces is completed. The hydrophobic hydration of nonpolar surfaces then initiates and a water monolyer is formed around the protein. The water bind to protein is then associates with the bulk water and hydration is completed (Fig. 4.10) (Damodaran 1996b). Water binding capacity (also called hydration capacity) of proteins defined as grams of water bound per gram of protein when a dry protein powder is equilibrated with water vapor at 90-95 % relative humidity (Damodaran 1996a). It is reported that the globular proteins bind about 0.2-0.5g water per gram of protein. Random coiled proteins such as gelatin, on the other hand, can bind almost 99 times of their weight of water due to the entrapment of water inside protein structure (Davidek et al. 1990).

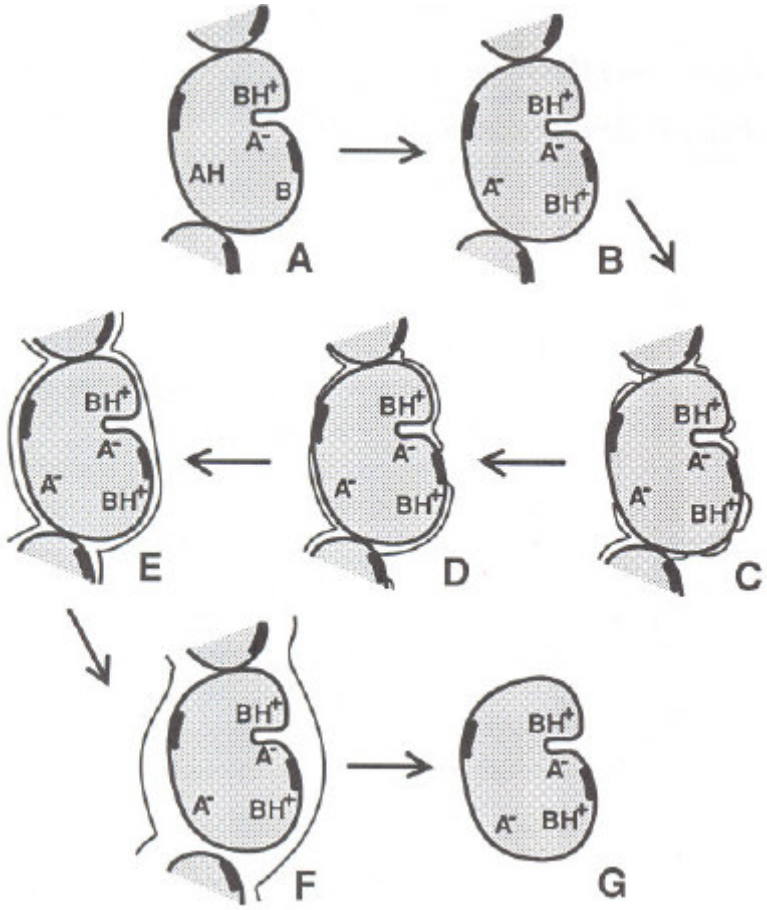


Figure 4.10. Protein hydration steps: (A) dry protein, (B) initial hydration at the sites of ionizable groups, (C) formation of water clusters near the polar and charged protein surfaces, (D) completion of hydration at the polar surfaces, (E) hydrophobic hydration at the nonpolar surfaces; completion of monolayer coverage, (F) bringing of water associated with protein with the bulk water, (G) complation of hydrodynamic hydration (Damodaran 1996a).

The water binding capacity of proteins are influenced by several environmental factors such as pH, ionic strength, type of salts in the medium, temperature and protein conformation (Damodaran 1996a, Sikorsky 1997). From these factors the pH is particularly effective on water binding. At isoelectric pH the proteins contain both positive and negative charges. Thus, the attraction formed between the positive and negative charges of different proteins increases protein-protein interactions. This minimizes water-protein interactions and causes coagulation and insolubilization. Thus, proteins show low hydration at their isoelectric point (pI). At pH values higher or lower than their pI, the proteins have a net negative or positive charge, respectively, and this

increases the repulsive forces among protein molecules. In this case, the proteins interact with water and hydration capacity increases significantly. Due to the ionization of the sulfhydryl and tyrosine residues, the water binding capacity of the proteins generally reaches the highest level at the pH 8-9 (Damodaran 1996a).

The presence of low concentrations of salts (<0,2 M) increase the hydration of proteins by binding of the hydrated salt ions weakly to charged groups on protein. In contrast, at high salt concentrations water-salt ions interactions block the water-protein interactions and this causes dehydration of the protein. The increase of temperature, also reduces the hydration by decreasing hydrogen bonding and hydration of ionic groups. Denaturation may also increase the water binding capacity by increasing the surface area to mass ratio with the exposure of some buried hydrophobic groups. However, if aggregation occurs, water binding capacity of denatured protein may decrease with the increased protein-protein interaction. It should be noted that most denatured food protein are insoluble (Davidek et al. 1990). However, the water binding capacity of these proteins is not significantly different from that of their native forms (Damodoran 1996a). This clearly shows that the water holding capacity is not directly related with solubility. Moreover, generally water-holding capacity of a protein is more important than water binding capacity for food products. The water-holding capacity refers to bound water, hydrodynamic water, and the physically entrapped water. It is related mainly with the protein structure and defined as ability of the protein to hold the water within the protein matrix (Sikorski 1997). There is a positive correlation with the water-holding capacity and water-binding capacity.

4.3.2. Solubility

Solubility of proteins depends on the equilibrium between protein-protein and protein-solvent interactions. High solubility of a protein increases its functionality and usage in the food production. Hydrophobic and ionic characteristics of the proteins are the major factors that affect the solubility. Hydrophobic interactions decrease the solubility because of the promotion of protein-protein interactions while ionic interactions increase the solubility by promoting protein-water interactions (Damodaran 1996a).

Proteins are classified into four categories according to their solubility as, (1) albumins; soluble in water at pH 6,6 (e.g. serum albumin, ovalbumin); (2) Globulins; soluble in dilute salt solution at pH 7,0 (e.g. glycinin, phaseolin); Glutelins; soluble only in acid (pH 2,0) and alkaline (pH 12,0) solutions (e.g. wheat glutelins); (3) Prolamins; soluble in 70% ethanol (e.g. zein, gliadins).

One of the most important factors affecting protein solubility is pH. When solubility is plotted against pH, generally a U shaped curve was obtained with minimum solubility at isoelectric point (Sikorski 1997). A majority of the food proteins are acidic proteins that exhibit minimum solubility at pH 4-5 and maximum solubility at alkaline pH.

Depending on the pH of a medium and pI of proteins, the ionic strength may also be highly effective on protein solubility. For example, at pH values lower than the pI, protein has a net positive charge. Under this condition, increase of the ionic strength of medium by addition of sodium chloride results the preferential binding of negatively charged chloride ions to protein. As the salt concentration was increased the net positive charge of protein and molecular repulsion reduces. Thus, protein solubility decreases since reduced molecular repulsion cause an increase in the hydrophobic interactions that promote protein-protein interaction. If pH of the medium is close to the pI of the protein, the increase of ionic strength by addition of salt, increases also the electronegativity of protein by preferential binding of chloride ions to positive charges of protein. This increases the molecular repulsion while destabilizing hydrophobic protein-protein interactions and solubility increases. On the other hand, when pH is above pI, despite the binding of the sodium and chloride ions to counter groups in protein, the net negative charge of protein maintains its solubility (Damodaran 1996b).

The effect of temperature on solubility of proteins depends on the temperature range. For example between 0 and 40 ^{o}C solubility of proteins, except highly hydrophobic proteins, increase with the increase of temperature. At higher temperatures unfolding of proteins expose the hydrophobic groups and cause aggregation and precipitation.

On the other hand, the organic solvents lower the permittivity of an aqueous medium. This increase intra- and intermolecular electrostatic forces, both the repulsive and attractive. The increased repulsive electrostatic interactions cause the unfolding of the protein and the promotion of H bonding and electrostatic attractions by low permittivity cause precipitation (Demodaran 1996a).

4.3.3. Interfacial Properties of Proteins

Most of the processed food products are two-phase systems such as emulsions or foams that are unstable without a suitable amphiphilic (or amphipathic) material between the surfaces of their two phases. Proteins containing both hydrophobic and hydrophilic groups are amphiphilic molecules that migrate spontaneously to an air-water interface or an oil-water interface (Damodaran 1996a). The highly viscoelastic film formed at an interface by proteins is more stable to mechanical disturbance than low-molecular weight surfactants.

Although all the proteins are amphiphilic, their surface-active properties may show difference. The differences in the surface active properties of the proteins are primarily attributed to differences in their conformations and differences in the physicochemical characteristics of their external surface (Damodaran 1996b). In fact these are the conformational stability/flexibility, adaptability of conformation to environmental changes and distribution of hydrophilic and hydrophobic groups on the protein surface. The major characteristic properties of surface-active proteins are; (1) ability to rapidly adsorb to an interface, (2) ability to rapidly unfold and reorient at an interface, (3) ability to form a strong cohesive and viscoelastic film at the interface that is stable to thermal and mechanical shocks. These characteristics of proteins greatly affect their emulsifying and foaming properties (Damodaran 1996a, Damodaran 1996b).

4.3.3.1. Emulsifying Properties

Emulsions are dispersions of one liquid in another (Walstra 1996). Emulsions have three components; oil, water and emulsifier. However, to form the emulsion it is essential to apply energy (mostly mechanical energy) and break up oil into small droplets. The energy needed to disperse and break up oil droplets is generally provided by intence agitation (Walstra 1996). If oil and water are dispersed through the application of energy without the use of a emulsifier, the system attempts to achieve the conformation of lowest free energy. The total energy can be minimized if the area of contact between the two liquits is kept minimized. This can initially be achieved by the formation of spherical oil particles. If there is no energy barrier to prevent coalescence, the system will continue to lower its total energy content by the formation of larger

droplets from smaller ones. After some time phase seperation occurs and emulsion is disturbed. The proteins used as emulsifier generally form a lipid around the lipid globules and with their electostatic charge and steric hindrance they prevent flocculation of lipid globules (Hu et al. 2003, Sikorsky 1997). In other terms coating of lipid droplets by protein provides an energy barrier to coalescence. Proteins capable of unfolding at water-lipid interface can serve as emulsifier (Damodaran 1996a). The proteins are very suitable for oil-in-water food emulsions. However, their limited solubility in oil prevents their use in water-in-oil emulsions. Some intrinsic and extrinsic factors that can effect the properties of protein stabilized emulsions are given in Table 4.3.

Table 4.3. Intrinsic and extrinsic factors effective on properties of protein-stabilized emulsions (Damodaran 1996a)

Intrinsic Factors	**Extrinsic Factors**
pH	Type of emulsification equipment
Ionic strength	Rate of energy input
Temperature	Rate of shear
Presence of low-molecular-weight surfactants	
Sugars	
Oil phase volume	
Type of protein	
Melting point of oil used	

The examples of emulsion-type products stabilized by proteins include milk, egg yolk, coconut milk, soy milk, butter, margarine, mayonnaise, spreads, salad dressings, frozen desserts, frankfurter, sausage, and cakes (Damodaran 1996a).

4.3.3.2. Foaming Properties

Food foams are dispersions of gas bubbles (mostly air) in a continuous liquid or semi solid phase (Sikorsky 1997). There are many processed foam-type foods such as whipped cream, ice cream, cakes, bread, soufflés, mousses and marshmallow. The unique textural properties of these products are due to tiny air bubbles. In foods the

main surface active agents that help in the formation and stabilization of the dispersed gas phases are proteins (Damodaran 1996a).

Generally, protein stabilized foams are formed by bubbling, whipping or shaking a protein solution. The foaming property of a protein refers to its ability to form a thin firm film at gas-liquid interfaces, so that large quantities of gas bubbles can be incorporated and stabilized (Damodaran 1996a). The volume of the gas bubble may make up 99% of the total foam volume whereas the contends of protein in food products is 0.1-10% (Sikorsky 1997).

The foams are stabilized by lowering the gas-liquid interfacial tension and formation of rupture-resistant, elastic protein film surrounding the bubbles. If the foams are not fixed by heat setting of the protein network, they may be destabilized by (1) drainage of the liquid from the intersheet space due to gravity, pressure or evaporation; (2) diffusion of the gas from the smaller to larger bubbles; (3) coalescence of the bubbles by rupture of the protein films (Sikorsky 1997). The environmental factors influencing the foam formation and stability are pH, salts, sugars, lipids, protein concentration.

The protein foams are more stable at isoelectric pH of protein if no insolubilization of the protein occurs. In isoelectric pH region lack of repulsive interaction produces favorable protein-protein interactions and formation of a good film at the interface. The lack of repulsion also increases the amount of protein absorbed to the interface (Damodaran 1996a, Damodaran 1996b). At pH other than pI, foamability of protein is good, but foam stability is poor.

Due to cross-linking of protein molecules and creation of films with better viscoelastic properties, divalent ions such as Ca^{+2} and Mg^{+2} improve foamability and foam stability. Addition of sucrose, lactose and other sugars and increase of the protein concentration improves foamability due to the increased viscosity of liquid phase. In contrast, lipids impair the foaming properties.

4.3.4. Flavor Binding

Flavor is one of the important characteristics of the sensory properties of the foods. Although proteins are odorless they can bind flavor compounds. Proteins bind flavor compounds tightly, retain them during processing of foods, and release them

during chewing. In dry conditions proteins bind flavors with van der Waals interactions, hydrogen bonding, and electrostatic interactions. In liquid or high moisture products, proteins bind flavor through hydrophobic regions on the protein surface. Oilseed proteins and whey proteins carry undesirable flavors and this limits their food applications (Damodaran 1996a). On the other hand, the flavor binding properties of proteins may be exploited by using them as carriers of desired flavors. For example, flavor binding property of proteins is particularly useful to produce meat-like flavor with meat analogues containing plant proteins (Damodaran 1996a).

4.3.5. Viscosity

The viscosity of a solution is related to its resistance to flow under an applied force (or shear stress). Viscosity or consistency of the products is very important for the consumer acceptance of several liquid and semisolid-type foods (e.g. soups, beverages). High-molecular-weight polymers such as proteins greatly increase viscosity. The viscosity behavior of proteins is affected by several variables including size, shape, protein-solvent interactions, hydrodynamic volume and flexibility in the hydrated state.

4.3.6. Gelation

A gel is an intermediate phase between a solid and a liquid and it is defined as a substantially diluted system which exhibits no steady state flow (Damodaran 1996a). They are made up by polymers cross-linked by covalent or noncovalent bonds that form a three-dimensional network which entrap water and other small molecular weight substances. In gels the proteins are transformed from “sol state” to “gel-like” state. Heating, divalent ions and enzymes are used to conduct this transformation and form gels. However, the heating is the most frequently used method to obtain protein gels. The gelation mechanism of heating involves two steps. The first step usually involves the dissociation of the quaternary structure of protein and the second step involves the unfolding of protein molecules (Sikorsky 1997). Proteins are transformed into a ‘progel’ state due to the denaturation by heating. At a progel state, some degree of protein polymerization has already occurred, so that a viscous liquid is formed. The denaturation of proteins and unfolding exposes functional groups such as hydrogen

bonding groups and hydrophobic sites capable to promote protein-protein interactions and causes the formation of a network. The gelation occurs by the cooling of the progels. The gels formed mainly by H bonding are reversible and turned to the progel state by reheating. However, the gels formed mainly by hydrophobic interactions are irreversible due to the temperature stability of the hydrophobic interactions (Damodaran 1996a). In some proteins heating to about 40 ^{o}C may cause gelation whereas some fish protein sols turn to gels slowly at 4 ^{o}C. For the gelation of ovalbumin, on the other hand, it is necessary to apply a two step heating, fisrt to 60-70 ^{o}C and then to 85 ^{o}C (Sikorsky 1997). The denatured proteins may form coagulum-type gels formed irreversibly by hydrophobic aggregation due to great number of apolar amino acid residues or translucent type gels formed mainly by H bonding due to the low levels of nonpolar amino acid residues in protein.

4.3.7. Dough Formation

Food proteins, especially wheat proteins, have ability to form a viscoelastic dough suitable for making bread and other bakery products. The formation of dough and its characteristics stem form proteins. Gluten in cereals is the major protein for dough formation. The dough structure is based on extensive three-dimensional network of gluten protein sub-units joined together by disulfide cross-links (Davidek et al. 1990). Gluten is a mixture of gliadin and glutenins proteins and its amino acid composition affects the functionality of gluten in the dough. The high glutamine and hydroxyl amino acid residues responsible for the gluten water binding properties whereas cysteine and cystine residues have functions in the polymerization of gluten proteins due to sulfhydryl-disulfide interchange reactions.

4.3.8. Antioxidant Properties of Proteins

Due to the health concerns related to the use of synthetic antioxidants, extensive studies have been carried out to find or develop safe and natural antioxidants. Many proteins including casein, soy proteins, ovalbumin, oilseed proteins, gliadin, zein, bovine serum albumin, yam dioscorin, lactoferrin, sericin, carnosine, etc have been reported to have an antioxidant activity (Rajalakshmi and Narasimhan 1996, Kouoh et

al. 1999, Kim et al. 2001, Hou et al. 2001, Hu et al. 2003). It was reported that amino acids show their antioxidative properties both as primary antioxidants or secondary antioxidants (Sakanaka et al. 2004). The proteins owe their antioxidant activity to their constituent amino acids. The antioxidant activity of aromatic amino acids such as tyrosine, phenylalanine and tryptophan and sulfur containing amino acids such as cysteine is due to their ability to donate protons to free radicals (Hu et al, 2003, Rajapakse et al. 2005, Je et al. 2004). On the other hand, the basic amino acids such as histidine, lysine and arginine and acidic amino acids such as aspartate and glutamate show their antioxidant activity by chelating metal ions (Saiga et al. 2003, Rajapakse et al. 2005). The reports of different workers show that histidine may behave as both a radical scavenger and a metal chelator due to its imidazole ring (Chen et al. 1996, Rajapakse et al. 2005). Thus, this amino acid may have a critical importance for the antioxidant activity of proteins. It is also reported that there is a close relationship between the hydrophobicity and antioxidant activity of peptides (Chen et al. 1995, Rajapakse et al. 2005, Saıga et al. 2003). In fact, many antioxidative peptides contain hydrophobic amino acid residues such as valine and leucine at the N-terminus (Kim et al. 2001). It seems that the hydrophobicity is important since it increases the interaction of protein with the lipids. Moreover, Hu et al. (2003) reported that the cationic characteristics of protein inhibit lipid oxidation due to the electrostatic repulsion of transition metals away from the lipid droplets.

The presence of some antioxidant amino acids is not the only factor that determines the antioxidative properties of proteins or peptides. The correct positioning in the peptide sequence is also a very important factor effective on antioxidant activity (Rajapakse et al. 2005, Chen et al. 1996). It was reported that the position of histidine, proline, leucine, and glutamic acid in the chains of antioxidative peptides is effective on their radical scavenging activities. For example, the peptides having proline at the N-terminus more effectively prevents oxidation of linoleic acid than peptides having proline at the C-terminus (Chen et al. 1996). Also, peptides having histidine residues at the N-terminus show higher metal chelating activity than peptides having histidine at the C-terminus (Chen et al. 1998).

By modification, it is possible to enhance the antioxidant activity of proteins. For example, it was reported that Maillard reaction with polysaccharides may increase the antioxidant activity of proteins by improving their hydrophilic/hydrophobic balance (Nakamura et al. 1998). The antioxidant activity of protein extracts may also be

increased by Maillard reaction of proteins with sugars. However, in this case, the increase in antioxidant activity is mainly due to the formation of Maillard reaction products having high antioxidant activity (Yoshimura et al. 1997, Alaiz et al. 1999, Nicoli et al. 1999). The antioxidant activity of proteins can also be increased by their hydrolysis with proteases (Chen et al. 1996, Chen et al. 1998, Pena-Ramos and Xiong 2002) or concentrated acid (Chen et al. 1995). Protein hydrolysates, containing peptides and amino acids have long been known to act as potent antioxidants. However, the degree of hydrolysis may be very important to obtain optimum antioxidant activity. It was reported hat the limited hydrolysis works better than the complete hydrolysis to increase the antioxidant activity, since it exposes the functional groups (amino acids or peptides) and increases protein-lipid interactions (Hwang et al. 2001). For enzymatic hydrolysis, the amino acid sequence of protein is highly effective on the antioxidant activity, since it effects the substrates produced by the protease enzyme used in hydrolysis (Chen et al 1995).

4.4. Modification of Proteins

The intentional modification of proteins is conducted to enhance the physicochemical and functional properties of proteins (Howell 1996). Food proteins have been modified since 5000 B.C. A classical example is enzymatic modification of milk proteins in yoghurt and cheese.

4.4.1. Chemical Modification

Chemical modification of proteins includes the derivatization of the amino acid side chains of proteins and hydrolysis of the peptide bonds (Howell 1996). Although chemical modification of amino acid side chains can improve functional properties of proteins, it can also impare the nutritional values and may create some toxic amino acid derivatives that cause regulation problems (Domadoran 1996a). The major amino acid side chains involved in chemical modifications and related chemical modifications in these groups were given in Table 4.4.

Table 4.4. The amino acid side chain groups and related modifications (Howell 1996)

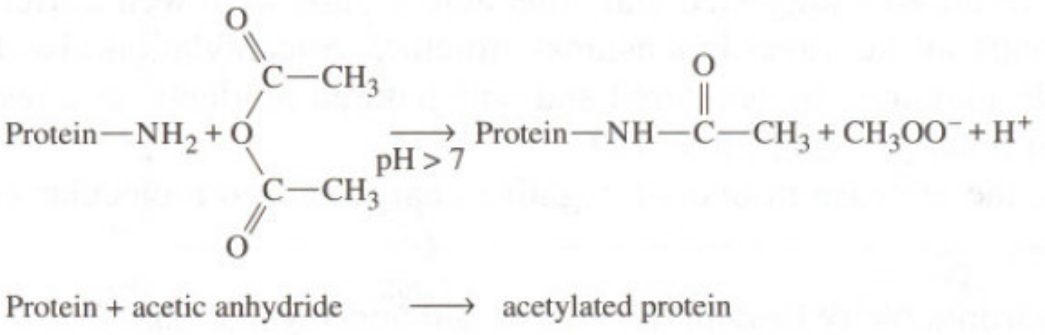
Side Chain	Chemical Modifications
Amino	Acylation, alkylation
Carboxyl	Esterification, amidation
Disulfide	Oxidation, reduction
Sulfhydryl	Oxidation, alkylation
Thioether	Oxidation, alkylation
Phenolic	Acylation, electrophilic substitution
Imidazole	Oxidation, alkylation
Indole	Oxidation, alkylation

4.4.1.1. Acylation

Acylation of proteins is conducted with acid anhydrides. Mono and dicarboxylic acids, e.g. acetic and succinic anhydrides, are commonly used as acylating agents. These chemicals react mainly with ε-amino groups of lysine. Also, they undergo limited reaction with the tyrosine phenolic groups.

Acylation with acetic anhydride is called acetylation, whereas acylation with succinic anhydrides is called succinylation (Fig. 4.11). Acetylation involves the covalent attachment of neutral acetyl groups to positively charged ε-amino groups. However, succinylation introduces anionic succinate residues to the ε-amino group and makes net charge of protein negative. This increases molecular repulsion and increase solubility (Howell 1996). However, succinylation impairs some functional propertied such as heat-gelling properties, foaming and emulsifying activity (Damodaran 1996a).

(a)



(b)

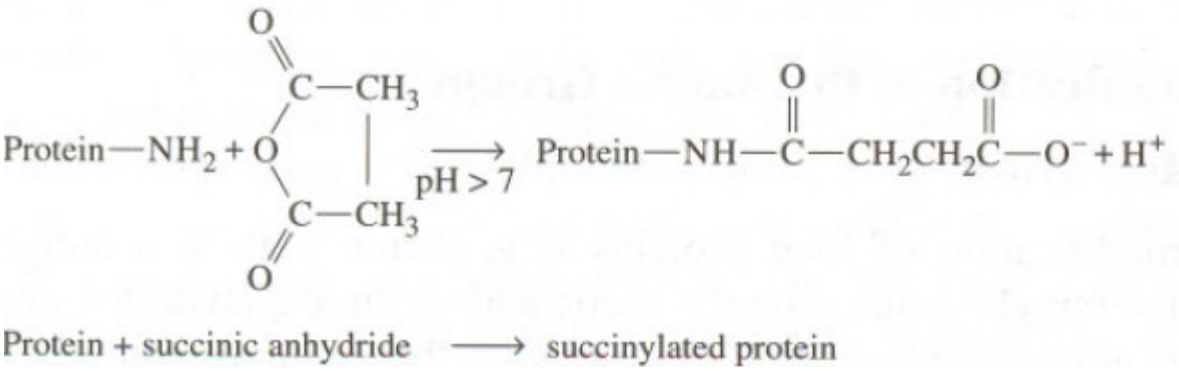


Figure 4.11. Acetylation (a) and succinylation (b) of proteins (Howell 1996)

4.4.1.2. Alkylation

The SH and amino groups can be alkylated by reacting them with iodoacetate or iodoacetamide (Fig. 4.12). Reaction with iodoacetate results in elimination of the positive charge of lysyl residue, and introduction of negative charges at both lysyl andf cyteine residues.

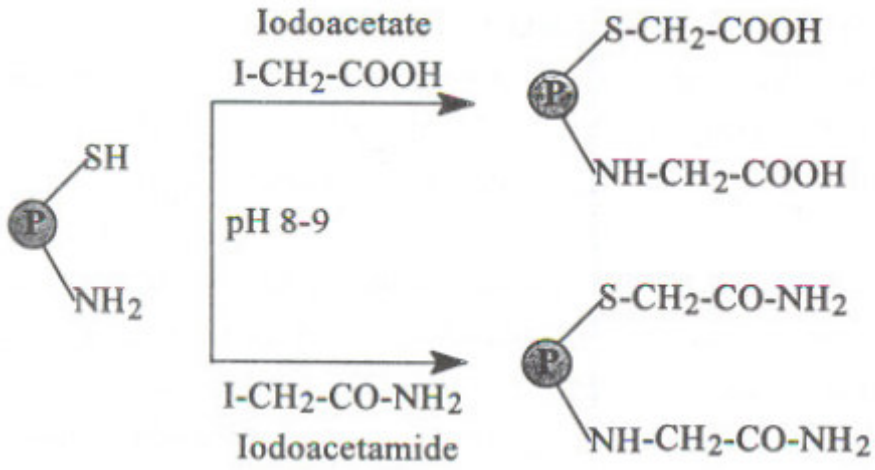


Figure 4.12. Alkylation with iodoacetate and iodoacetamide (Damodaran 1996a)

The increase of the electronegativity of iodoacetate-treated protein may increase the solubility of protein. On the other hand, reaction with iodoacetamide eliminates only the positive charges. Reaction of iodoacetamide also effectively block sulfhydryl groups and prevent disulfide-induced protein polymerization (Damodaran 1996a).

4.4.1.3.Esterification

The acylation is the most widely used method of chemical modification of food proteins. However, since it modifies an essential amino acid (lysine) side chain it causes impairment of the nutritional values of protein. Thus, modification of the β- and γ-carboxyl groups of nonessential amino acids aspartic and glutamic acid by esterificaiton is more acceptable than the acylation.

To prepare the esters, carboxyl groups of protein are treated with methanol or ethanol in the presence of an acid catalyst (Howell 1996)(Fig. 4.13). Esterification blocks the negatively charged carboxyl groups, yielding a protein with an increased isoelectric point (pI) or net positive charge.

The esterification modifies the functional properties of proteins. For example, ethyl-esterified β-lactoglobulin exhibits superior emulsion stability and oil adsorption properties at the oil-water interface than its native form (Howell 1996). The esters are stable at acid pH, but are readily hydrolyzed at alkaline pH (Damodaran 1996a).

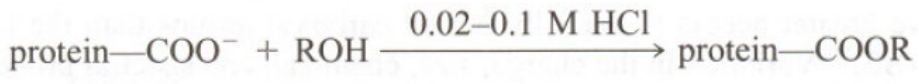


Figure 4.13. Esterification of protein (Howell 1996)

The esterification modifies the functional properties of proteins. For example, ethyl-esterified β-lactoglobulin exhibits superior emulsion stability and oil adsorption properties at the oil-water interface than its native form (Howell 1996). The esters are stable at acid pH, but are readily hydrolyzed at alkaline pH (Damodaran 1996a).

4.4.1.4.Amidation

Similar to esterification, amidation also targets nonessential acidic amino acids. Carboxyl groups of aspartate and glutamate can be converted to asparagines and glutamine, respectively, by reacting with a nucleophilic reagent (such as amine) and a water soluble carbodiimide (WSC) (Fig. 4.14).

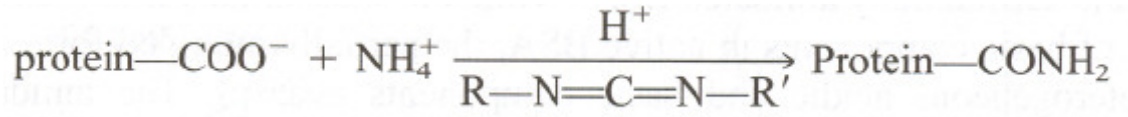


Figure 4.14. Amidation of proteins (Howell 1996)

Carbodiimides characterized by their unsaturation show addition reaction with a range of organic functional groups. For example, by this way essential amino acids may be attached covalently to proteins (Howell 1996). By amidation it is also possible to retard the thermogelling of egg albumen.

4.4.1.5.Acid hydrolysis

The mild hydrolysis is applied to obtain high molecular weight products with increased functionalities such as solubility, foaming and emulsification. Mild acid hyrolisis results in low levels of peptide bond hydrolysis (7 %) accompanied by deamidation (10-20%) that results in release of aspartic acid and ammonia (Howell 1996). On the other hand, high degree of acid hydrolysis is applied to obtain protein hydrolisates. Seasonings containing amino acids obtained by acid hydrolysis of proteins are important commodities in world (Davidek et al. 1990). Currently, the most frequently used raw materials to obtain protein hydrolisates are soybean meal and wheat gluten. The process is generally conducted with 20% hydrochloric acid at 110 ^{o}C for several hours. The crude hydrolisate was then neutralized with sodium hydroxide or sodium carbonate and it is filtered and stored for some time to improve its organoleptic properties before it is used in food applications (Davidek et al. 1990). The acidic hydrolysis causes the loss of some essential nutrients. For example, even dilute acids may cause the total loss of tryptophan.

4.4.1.6.Alkaline Hydrolisis

Deamidation and hydrolysis of peptide bonds by alkali treatment is used generally to isolate plant proteins. The extraction is conducted by use sodium hydroxide for several hours between 50 and 80 ^{o}C. Alkali treatments even at moderate temperatures cause racemization of all optically active amino acids and formation of some nondigestable D-isomers (Davidek et al. 1990). Serine and aspartic acid residues are the

residues that undergo the greatest racemization. The alkali treatment also causes the destruction of lysine and cysteine, forms some crosslinked amino acids such as lysinoalanine and leads the formation of some possibly toxic compounds. Thus, protein hydrolizates are now obtained under mild conditions by using sodium bicarbonate, bisulphide and neutral solutions in place of sodium hydroxide (Davidek et al. 1990).

4.4.1.7. Phosphorylation

Phosphate groups can be covalently attached to proteins to increase their negative charges (Fig. 4.15). Inorganic phosphate can be bound to proteins either by O- or N-esterificaiton reactions. In O- esterification, during the formation of C-O-P bond derivatives, inorganic phosphate react with the hydroxyl groups of serine, threonine and tyrosine and form phosphoserine, phosphotreonine and phosphotyrosine, respectively. On the other hand, in the C-N-P derivatives produced by N- esterification, the inorganic phosphate combines with the amino group of lysine, imidazole group of histidine and guanidino group of arginine (Howell 1996). The phosphorylation greatly increases the electronegativity of proteins. However, the use of inorganic phosphate in phosphorylation produces some protein cross-links that reduce the solubility. Thus, the use of phosphorylation agents such as sodium trimetaphosphate which does not cause cross-linking is suggested. Phosphorylation enhances viscosity, water absorption, gelation and emulsification. Since phosphorylated proteins are highly sensitive to calcium ion induced coagulation, they may be very suitable for simulated cheese type products (Damodaran 1996a). The nitrogen-bound phosphate derivatives are less acid stable than the oxygen-bound derivatives. Thus, the digestibility of proteins modified by nitrogen- bounding of phosphate is not impaired significantly.

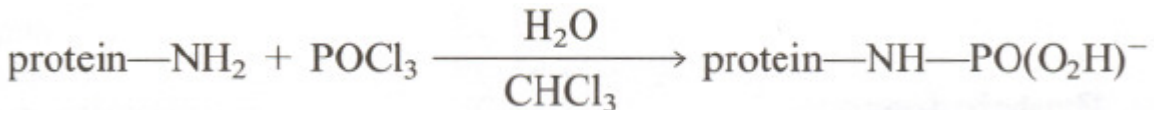


Figure 4.15. Phosphorylation of proteins (Howell 1996)

4.4.2. Enzymatic Modifications

Enzymes (mostly proteases) are used to modify the functional properties of proteins such as viscosity, gelability, moisture-retaining capacity, dispersibility, elasticity, and foam stability to different degree (Davidek et al. 1990).

In food industry, the limited hydrolysis of proteins by proteases found many applications such as solubilization of denatured proteins, maintenance of protein solubility in acid media (e.g. in carbonated acid beverages), improvement of digestibility (foodstuffs for children, diet-followers, sportsmen, old people, components of animal fodder), decomposition of proteins having undesirable properties, tenderization of meet and chill proofing of beer (Davidek et al. 1990).

A high degree of protein hydrolysis, on the other hand, is applied in the preparation of protein hydrolisates for parenteral nutrition, preparation of seasonings etc.

Depending on the type of protein and the type of modification different protease enzymes from animal, plant or microbial origin may be used in the enzymatic modification of proteins. For example, to obtain protein hydrolysates containing small and nonbitter peptides it is suggested to use aminopeptidases that cleave off dipeptides from the amino terminal. The main enzymes used in protein modification include FDA approved plant origin enzymes including papain from papaya, bromelain from pineapple and ficin from ficus (Howell 1996). Malt proteases may also be used for the modification of proteins.

In addition to hydrolysis of peptide bonds of proteins it is possible to promote enzymatic cross-linking, deamidation or phosphorylation of proteins. For example, by using transglutaminase, it is possible to conduct protein cross-linking or introducing essential amino acids to proteins (De Jong and Koppelman 2002, Howell 1996). On the other hand, peptidoglutaminase catalyses the deamidaiton of proteins by hydrolysis of amide of glutamine residues, whereas protein kinase catalyses the phosphorylation of proteins (Howell 1996).

CHAPTER 5

MATERIALS AND METHODS

5.1. Materials

Dried chick-peas and kidney-beans (Migros Nohut (koçbaşı), Şok Fasulye (dermason)) were purchased from a supermarket in Izmir (Turkey). The dialysis tubing (12000 MW, prepared as described in the product manual), bovine serum albumin (fraction V), DEAE-cellulose (fast flow column, prepared as described in product manual), insoluble PVPP (polyvinylpolypyrrolidone), ABTS (2,2-Azino-bis-(3-Ethylbenz-Thiazoline-6-Sulfonic acid)), linoleic acid (99 %), Tween 20 were purchased from Sigma Chem. Co. (St. Louis, Mo., USA). Ammonium sulfate (for biochemistry) was purchased from Merck (Darmstadt). Trolox, Ferrous chloride tetrahydrate was purchased from Fluka (Switzerland). Ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4"-disulfonic acid Monosodium salt) was purchased from Fluka (USA). Sericin (Silk Biochemical Co. Ltd) was kindly donated by Assistant Professor Dr. Oğuz Bayraktar from Izmir Institute of Technology.

5.2. Preparation of Samples

To prepare the heat treated samples, the legumes were first rehydrated in distilled water at 12 h at room temperature. The samples were than heat treated at 90^{o} C for 20 minutes. On the other hand, for the preparation of thermally processed samples, the legumes were put into flasks containing distilled water and thermally processed at 121^{o} C for 20 minutes. The treated samples were processed immediately to acetone powder.

5.3. Preparation of Acetone Powders

To remove phenolic compounds and lipids, acetone powders were used as source of protein extracts. For the preparation of acetone powders, untreated, heat-treated or

thermally-processed chick-peas or kidney-beans (prepared by rehydration of 50 g dry samples) were homogenized in a Waring blender for 3 min with 200 mL cold acetone. The slurry obtained was filtered under vacuum from Buncher funnel containing a Whatman No:1 filter paper and the solid residue remained on the filter paper was collected. The homogenization with 200 mL cold acetone and filtration were then repeated for two more times for the collected residue and the powder, left overnight to evaporate the acetone, was stored at -18 ^{o}C until used for protein extraction.

5.4. Extraction and/or Modification Methods

5.4.1. Preparation of Crude Protein Extracts of Heat Treated or Thermally Processed Chick-peas or Kidney-beans

The heat treatment of samples was applied for the inactivation of enzyme lipoxygenase, whereas thermal processing was applied both for lipoxygenase inactivation and modification of antioxidant activity of proteins. To prepare the crude protein extracts from heat treated or thermally processed chick-peas or kidney-beans the extraction method given by Genovese and Lajolo (1998) was applied by major modifications. Briefly, 20 g acetone powder, 0.5 g insoluble PVPP and 180 ml distilled water were mixed and extracted with a magnetic stirrer for 2 hours at room temperature. The extract was then filtrated from a cheese-cloth (4 layers) to collect the filtrate and the cake was discharged. The filtrate was then centrifuged for 30 min at 15000 g (4 ^{o}C) and clarified. Half of the clarified supernatant was dialyzed for 72 h (48 h against 5 x 2 L distilled water and 24 h against 3 x 2 L deionized water) at 4 ^{o}C, whereas the remaining half was incubated for the same period at the same temperature without application of dialysis. At the end of dialysis (or incubation without dialysis) the extracts were clarified by centrifugation for 15 min at 4500 g (4 ^{o}C) and stored at -18 ^{o}C after they were lyophilized. The lyophilization was conducted by using a freeze drier (Labconco, FreeZone, 6 liter, Kansas City, MO, USA) working between -44 and -47 ^{o}C collector temperature and 50 x 10^{-3} and 100 x 10^{-3} mBar vacuum. The sample container volume was two to three times the sample volume.

5.4.2. Preparation of Crude Protein Extracts of Chick-peas by Hot Acidic Hydrolysis

The hot acidic hydrolysis was applied mainly for the modification of antioxidant activity of proteins. For this purpose, 4 g acetone powder from rehydrated chick-peas was suspended in 65 ml deionzied water and the total volume of the suspension was adjust to 130 ml with 3 N HCl. The extract was then heated to 85 ^{o}C and maintained at this temperature for 10 or 30 minutes under continuous stirring. After heating, the extract was cooled to room temperature in an ice water bath and its pH was brought to neutrality by addition of 6 N NaOH. The final volume of this extract was then made up 155 mL, it was further stirred (30 or 50 min for 30 and 10 min heated samples, respectively) at room temperature and clarified by centrifugation for 30 min at 15000 g (4 ^{o}C). Half of the clarified supernatant was dialyzed for 24 h (against 3 x 2 L deionized water) at 4 ^{o}C, whereas the remaining half was incubated for the same period at the same temperature without application of dialysis. At the end of dialysis (or incubation without dialysis) the extracts were clarified by centrifugation for 15 min at 5000 g (4 ^{o}C) and assayed for protein and antioxidant activity.

5.4.3. Preparation of Crude Protein Extracts of Chick-peas Obtained by Hot Extraction Conducted at Different pH Values

5.4.3.1. Hot Extraction Conducted Close to Neutrality

The hot extractions conducted close to neutrality aimed mainly the inactivation of enzyme lipoxygenase. On this purpose, 4 g acetone powder was suspended in 130 mL deionzied water. The extract which pH was almost 6.5 was then heated to 85 ^{o}C and maintained at this temperature for 30 or 60 minutes under continuous stirring. After heating, the extract was cooled to room temperature in an ice water bath and its volume was made up 150 mL. For extract heated for 30 min, an additional 30 min stirring was applied at room temperature whereas the 60 min heated extract was used without further stirring. The extracts stirred for total of 60 min were then clarified by centrifugation for 30 min at 15000 g (4 ^{o}C). Half of the clarified supernatant was dialyzed for 24 h (against 3 x 2 L deionized water) at 4 ^{o}C, whereas the remaining half was incubated for

the same period at the same temperature without application of dialysis. At the end of dialysis (or incubation without dialysis) the extracts were clarified by centrifugation for 15 min at 5000 g (4 ^{o}C) and assayed for protein and antioxidant activity.

5.4.3.2. Hot Extraction Conducted at Acidic or Basic pH Values

The hot extractions conducted at extreme pH values aimed both the modification of proteins and increase of extraction yield of antioxidant proteins. In these extractions, 4 g acetone powder was suspended in 100 mL deionzied water. The pH of the extract was then set to 2.5 (with 0.1 M HCl) or 9.5 (with 0.1 M NaOH) and it was heated to 85 ^{o}C and maintained at this temperature for 30 min under continuous stirring. The extract was then cooled to room temperature in an ice water bath and its volume was made up 150 mL. After an additional 30 min stirring at room temperature, the extract was clarified by centrifugation for 30 min at 15000 g and 4 ^{o}C. Half of the clarified supernatant was then dialyzed for 24 h (against 3 x 2 L deionized water) at 4 ^{o}C, whereas the remaining half was incubated for the same period at the same temperature without application of dialysis. At the end of dialysis (or incubation without dialysis) the extracts were clarified by centrifugation for 15 min at 5000 g (4 ^{o}C) and assayed for protein and antioxidant activity.

5.5. Partial Purification of Crude Protein Extracts with Ammonium Sulfate Precipitation and Dialysis

For partial purification, solid $(NH_4)_2SO_4$ was added slowly to undialyzed crude protein extracts at 4 ^{o}C up to 90 % saturation. The mixture was stirred slowly for 1.5 h at 4 ^{o}C and the precipitate formed collected by 30 min (or 45 min) centrifugation at 15000 g (or 4500g) and 4 ^{o}C was dissolved in 20 ml distilled water. The extract was then dialyzed for 24 h (or 36 h) at 4 ^{o}C (against 3 or 4 x 2L of distilled or deionized water), clarified by centrifugation for 30 min at 15000 g (4 ^{o}C) and then lyophilized and stored at -18 ^{o}C.

5.6. Partial Purification of Antioxidant Proteins by Dialysis and DEAE-cellulose Column Chromatography

5.6.1. Purification of Antioxidant Proteins from Dialyzed Crude Protein Extracts of Heat Treated or Thermally Processed Chick-peas

For the partial purification of heat treated or thermally processed chick-pea proteins, crude protein extract was prepared by suspending 10 g acetone powder and 0.25 g PVPP in 90 ml distilled water. After 2 h stirring at room temperature, the mixture was filtered from cheese cloth (4 layers), clarified by centrifugation for 30 min at 15000 g (4 ^{o}C) and dialyzed for 72 h (48 h against 5 x 2 L distilled water and 24 h against 3 x 2 L deionized water) at 4 ^{o}C. Following dialysis the extract was centrifuged for 15 min at 4500 g (4 ^{o}C) and loaded onto DEAE-cellulose column (2.4 cm diameter, 10 cm height) previously equilibrated with 0.01 M pH 7.00 Na-phosphate buffer. The washing of the column was conducted by 300 mL of equilibration buffer and the column was then eluted with a continuous linear gradient of 0-1.5 M NaCl prepared in 0.01 M pH 7.00 Na-phosphate buffer. Fractions (5 mL) collected from the column were assayed for their antioxidant activity against ABTS radical as described in section 5.7, and the inhibition period of tests was shortened to 2 min to complete the measurements of all fractions as soon as possible and prevent possible changes in the antioxidant properties of proteins. The protein content of the fractions, on the other hand, was monitored by measuring absorbance value at 280 nm.

5.6.2. Partial Purification of Antioxidant Proteins from Crude Protein Extracts of Chick-peas Obtained by Hot Extraction

To purify antioxidant proteins obtained by hot extraction, 4 g acetone powder was suspended in 130 mL deionzied water. The extract was then heated to 85 ^{o}C and maintained at this temperature for 30 minutes under continuous stirring. After heating, the extract was cooled to room temperature in an ice water bath, its volume was made up 150 mL and it was further stirred for 30 min at room temperature. The extract was then clarified by centrifugation for 30 min at 15000 g (4 ^{o}C), incubated for 24 h at 4 ^{o}C and one more centrifuged for 15 min at 4500 g (4 ^{o}C). The crude protein extract was

then loaded onto a DEAE-cellulose column (2.4 cm diameter, 10.0 cm height) previously equilibrated with 0.01 M pH 7.00 Na-phosphate buffer. The washing of the column was conducted by x mL of equilibration buffer and the column was then eluted with a continuous linear gradient of 0-1.5 M NaCl prepared in 0.01 M pH 7.00 Na-phosphate buffer. Fractions (5 mL) collected from the column were assayed for their antioxidant activity against ABTS radical and protein as described in section 5.6.1.

5.7. Determination of Antioxidant Activity against ABTS Radical

The antioxidant activity against ABTS radical was determined as described in Re et al. (1999). The ABTS was dissolved in distilled water, oxidized by potassium persulfate to form ABTS radical and then diluted with 5 mM pH 7.4 phosphate buffer containing 150 mM NaCl (PBS). The reaction mixture for the measurements was prepared by mixing 0.1 mL protein extract and 1.9 mL ABTS radical solution (initial absorbance at 734 nm was almost 0.700). The discoloration of dark blue colored ABTS radical by the antioxidant protein was monitored at 734 nm for 15 min. All measurements were performed in triplicate The antioxidant capacities of lyophilized protein preparations were determined by dividing the area of their % inhibition of ABTS radical / concentration (μg/reaction mixture) ratio vs. period of inhibition test (in 1, 6 or 15 min) curves with that area of the same curve of the standard antioxidant Trolox. The value determined by this calculation is called AUC (Area Under the Curve) value and it represents the antioxidant capacity as μmol Trolox per mg of lyophilized protein preparation. Bovine serum albumin and sericin were used as standard proteins for comparison. During purification studies the antioxidant activity of proteins were given as Trolox and Tyrosine equivalents (see standard curves in Appendixes A and B).

5.8. Determination of Fe^{+2} Chelating Capacity

The Fe^{+2} chelating capacity of protein extracts were determined as described in Rajapakse et al. (2005). Briefly, 2 mL protein solution was mixed with 0.1 mL, 1mM $FeCl_2.4H_2O$ solution. After 30 min incubation at room temperature, 0.1 mL, 0.5 mM ferrozine was added into mixture and its absorbance was read at 562 nm after 10 minute incubation. The formation of blue color indicates weak Fe^{+2} chelating capacity whereas

the lack of any blue color development shows the strong Fe^{+2} binding. The percent Fe^{+2} chelating capacity of sample was determined by using the following formula; A_1-A_2/A_3 x 100, where A_1 is the final absorbance of the treated sample at 562 nm, A_2 is the original absorbance of the untreated sample at 562 nm and A_3 is the absorbance of blank obtained by treating deionized water in place of sample. The Fe^{+2} chelating capacity of samples were given as EDTA equivalents (µmol) per mg of lyophilized protein preparation (See standard curve in Appendix C). All measurements were performed in triplicate.

5.9. Determination of Lipoxygenase Activity

In this study the enzyme lipoxygenase was used as an indicator for the determination of suitable heat treatment periods of chick-peas and kidney-beans. The presence of this enzyme in protein extracts intended to be used as antioxidant is undesirable since the enzyme is responsible from lipid oxidation. During preliminaries to determine a suitable heating condition for inactivation of enzyme in chick-peas or kidney-beans the enzyme extract was prepared by homogenizing 10 g of heat treated or unheated (control) sample with 50 mL distilled water in a Waring micro blender for 1 min. A sample taken from the extract was then clarified by centrifugation for 15 min at 15 000 g (4 ^{o}C) and used in test of enzyme activity. The activity of lipoxygenase was determined spectrophotometrically by slightly modifying the method described in Yemenicioglu (2002). The reaction mixture was formed by mixing 10 µL of clear enzyme extract, 2.95 mL 0.05 M, Na-phosphate buffer (pH 7.0) and 0.05 mL linoleic acid solution prepared with Tween 20 as described in Rackis et al (1972). The activity of enzyme monitored at 234 nm and 30 ^{o}C constant temperature was determined from the slope of the initial linear portion of absorbance vs. time curve.

5.10. Determination of Protein Content

Protein was determined by the Lowry method by using bovine serum albumin (BSA) as standard (see Appendices D) (Harris 1987).

CHAPTER 6

RESULTS AND DISCUSSIONS

6.1. Studies with Crude Protein Extracts of Heat Treated or Thermally Processed Chick-peas or Kidney-beans Obtained by Water Extraction

Since this work aimed studying the antioxidant activity of proteins, acetone powders were used to obtain phenolic free preparations. In studies with heat-treated or thermally processed chick-peas and kidney-beans, PVPP, an insoluble phenolic scavenger, was also employed during extraction to ensure the complete elimination of residual phenolics. On the other hand, the heat treatment (20 min at 90 ^{o}C) conditions were optimized by using the enzyme lipoxygenase as an indicator. Since this enzyme is responsible from the oxidation of lipids to hydroperoxides it should not exist in preparations intended to be used as antioxidant. The thermal processing (20 min at 121 ^{o}C) was applied mainly to modify the antioxidant activity of proteins, but it also inactivates the lipoxygenase enzyme.

6.1.1. Protein Content of Lyophilized Crude Protein Extracts of Heat-Treated or Thermally Processed Chick-peas and Kidney-beans

Chick-peas and kidney-beans contain almost 27 % and 22 % protein, respectively (WEB_1 2005, WEB_2 2005). In legumes, almost ~70 % the proteins consist of water insoluble globulins whereas the remaining protein consist of water soluble albumins (Genovese and Lajolo 1998, Vioque et al. 1999). Since extractions in this work were conducted with water, the proteins discussed in this study are mainly water soluble albumins.

As seen in Table 6.1, lyophilized crude chick-pea preparations contained significantly higher protein than lyophilized crude kidney-bean preparations. The thermal processing increased the protein content of undialyzed lyophilized chick-pea extracts slightly. However, a slight reduction was observed in the protein content of

undialyzed lyophilized kidney-bean extracts by the thermal processing. The application of dialysis caused an increase in protein/nonprotein substances ratio of lyophilized chick-pea preparations. In fact, in these extracts almost half of the lyophilized preparation was protein. In contrast, the dialysis did not affect the protein content of lyophilized kidney-bean preparations significantly. During dialysis, low molecular substances are removed from the extracts. The loss of some substances also occurred due to insolubilization and consequent separation in centrifugation applied after dialysis. For example, in heat treated chick-peas and kidney-beans, dialysis reduced the amounts of lyophilized dry powders almost 49 and 79 %, respectively. However, the increased protein content of dialyzed lyophilized chick-pea extracts indicates that the separated substances in these extracts are mainly non-protein substances. The soluble solids in the preparations other than the proteins may be polysaccharides such as soluble fractions of starch and pectin and simple sugars. Also, it is possible that an important part of the soluble solids exist as protein-polysaccharide complexes (Genovese and Lajolo 1998, Baldwin 2001).

Table 6.1. Protein contents of lyophilized preparations obtained from crude protein extracts of heat treated or thermally processed chick-peas and kidney-beans

Source of Protein	**Protein concentration in lyophilized preparation (mg protein /mg lyophilized preparaiton)**			
	Heat treated		**Thermally processed**	
	undialyzed	**dialyzed**	**undialyzed**	**Dialyzed**
Chick-peas	0.26	0.51	0.34	0.51
Kidney-beans	0.19	0.22	0.15	0.18

6.1.2 Antioxidant Activity of Lyophilized Crude Protein Extracts of Heat Treated or Thermally Processed Chick-peas and Kidney-beans against ABTS Radical

The results of inhibition tests for lyophilized crude protein extracts obtained from heat treated or thermally processed chick-peas and kidney-beans showed the presence of

antioxidants in these sources (Table 6.2. and 6.3.). During tests, the inhibitions occurred fast in the first minute. After that, the inhibition slowed down through the 6 min, but continued slowly up to 15 min or more. Thus, the absorbance values of ABTS radical solutions were monitored for 15 min and inhibition / concentration ratios of preparations were evaluated for 1, 6 and 15 min separately.

As given in Figure 6.1 and 6.2, at the end of 15 min inhibition test, chick-pea and kidney bean preparations showed higher antioxidant activity than the BSA protein against ABTS radical. By comparing the AUC values given in Table 6.4, it was determined that the lyophilized chick-pea and kidney bean protein preparations have 2 to 2.5 and 1.1 to 2 fold higher antioxidant capacity than BSA, respectively (Table 6.4). The BSA is accepted as one of the antioxidant proteins that have some important roles in the antioxidant mechanism of living cells (Kouoh et al. 1999). Thus, greater antioxidant activity of lyophilized chick-pea or kidney bean crude proteins compared to BSA showed the good potential of these plant sources for extraction of antioxidant proteins.

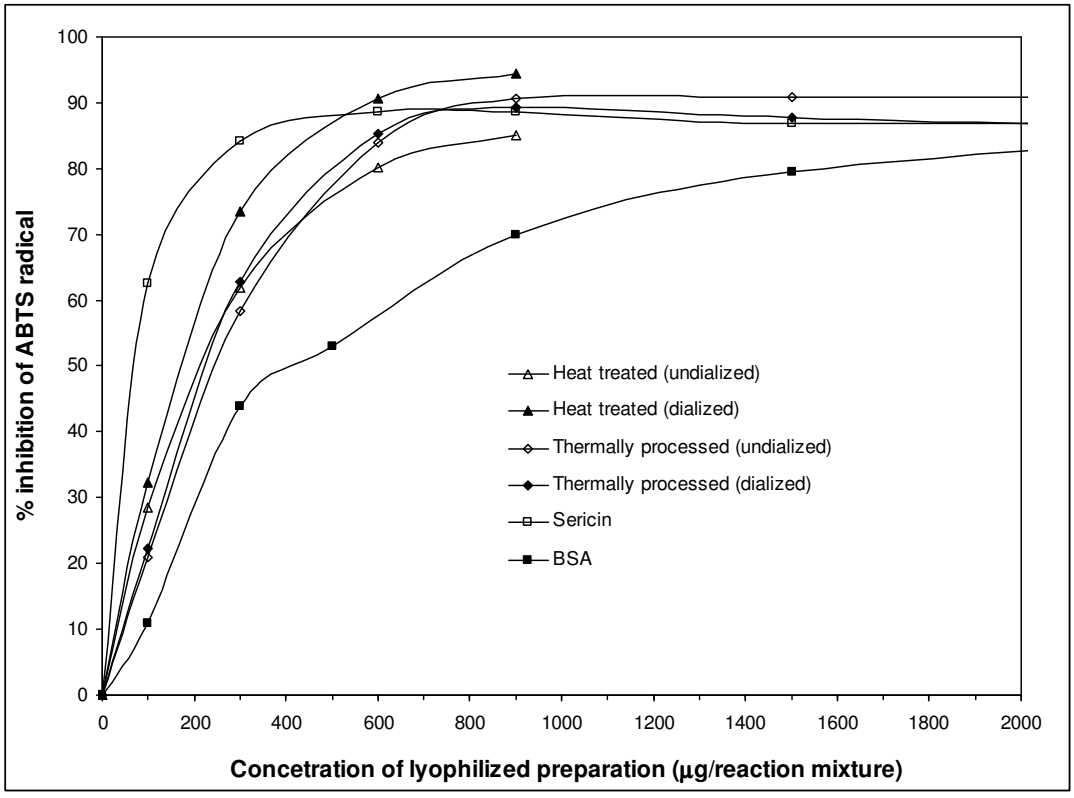


Figure 6.1. Inhibition of ABTS radical by different lyophilized crude protein preparations of heat treated or thermally processed chick-peas (for 15 min inhibition test)

Table 6.2. Inhibition of ABTS radical by lyophilized crude protein preparations of heat treated or thermally processed chick-pea

Sample	Concentration (µg/reaction mixture)	% inhibition of ABTS radical		
		1 min	6 min	15 min
Heat treated (undialyzed)				
	100	13.9	24.2	28.6
	300	30.9	52.2	62.0
	600	47.9	71.5	80.3
	900	58.2	79.4	85.1
Heat treated (dialyzed)				
	100	22.0	29.4	32.4
	300	49.1	66.0	73.6
	600	67.7	84.7	90.5
	900	77.9	91.6	94.5
Thermally processed (undialyzed)				
	100	12.8	18.7	20.9
	300	34.0	51.7	58.3
	600	53.4	75.4	83.9
	900	68.2	86.7	90.6
	1500	76.9	92.0	90.9
	3000	85.6	92.7	90.9
Thermally processed (dialyzed)				
	100	15.3	21.0	22.3
	300	40.2	56.2	62.9
	600	61.6	80.3	85.3
	900	73.0	87.2	89.4
	1500	77.8	88.3	87.7
	3000	83.6	87.1	85.2

Table 6.3. Inhibition of ABTS radical by lyophilized crude protein preparations of heat treated or thermally processed kidney-beans

Sample	Concentration (µg/reaction mixture)	% inhibition of ABTS radical		
		1 min	6 min	15 min
Heat treated (undialyzed)				
	20	0.71	1.7	2.3
	100	7.8	12.3	13.4
	300	24.5	36.9	42.2
	600	43.7	62.1	71.5
	900	57.7	76.2	82.9
Heat treated (dialyzed)				
	20	0.5	2.2	3.3
	100	8.9	14.1	15.9
	300	28.9	43.5	50.6
	600	46.5	68.6	77.4
	900	53.9	74.5	81.4
	1500	64.5	82.1	86.6
	3000	75.3	85.2	85.8
Thermally processed (undialyzed)				
	100	7.4	9.4	9.9
	300	21.6	31.2	35.7
	600	37.0	55.2	64.4
	900	48.9	68.5	77.2
	1500	58.1	74.0	78.4
	3000	72.4	80.4	78.6
Thermally processed (dialyzed)				
	100	6.9	11.2	11.7
	300	19.4	30.7	35.1
	600	32.6	50.1	57.9
	900	44.0	63.8	71.4
	1500	61.2	82.3	85.8
	3000	76.8	88.6	85.8

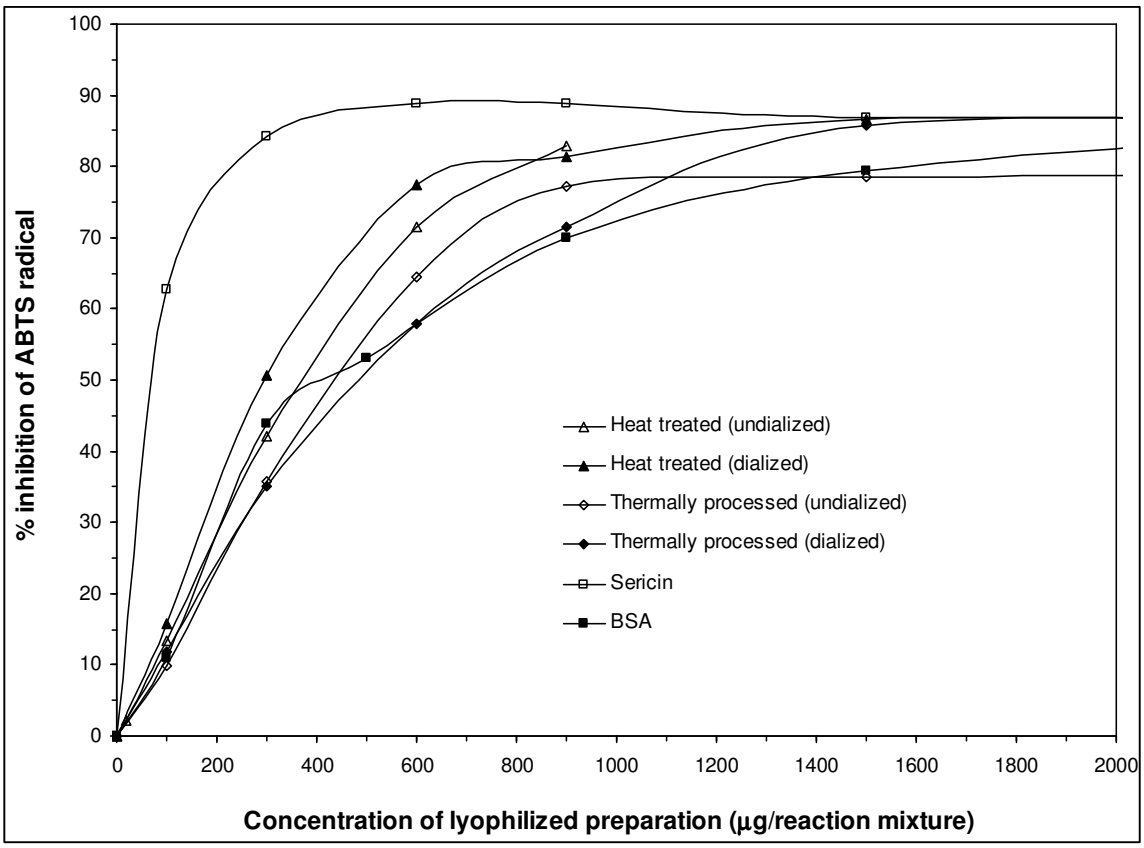


Figure 6.2. Inhibition of ABTS radical by different lyophilized crude protein preparations of heat treated or thermally processed kidney-beans (for 15 min inhibition test)

On the other hand, both lyophilized crude chick-pea and kidney-bean preparations showed lower antioxidant activity than sericin (Figure 6.3 and 6.4). However, it should be noted that the standard proteins, BSA and sericin, used in this study were highly pure, whereas lyophilized preparations' protein content changed between 15 to 51 %. These comparisons also clearly showed the greater antioxidant activities of lyophilized crude chick-pea proteins than the lyophilized crude kidney-bean proteins. The protein contents of chick-pea preparations were also higher. Thus, it seems that the greater antioxidant activity is related with the higher protein content.

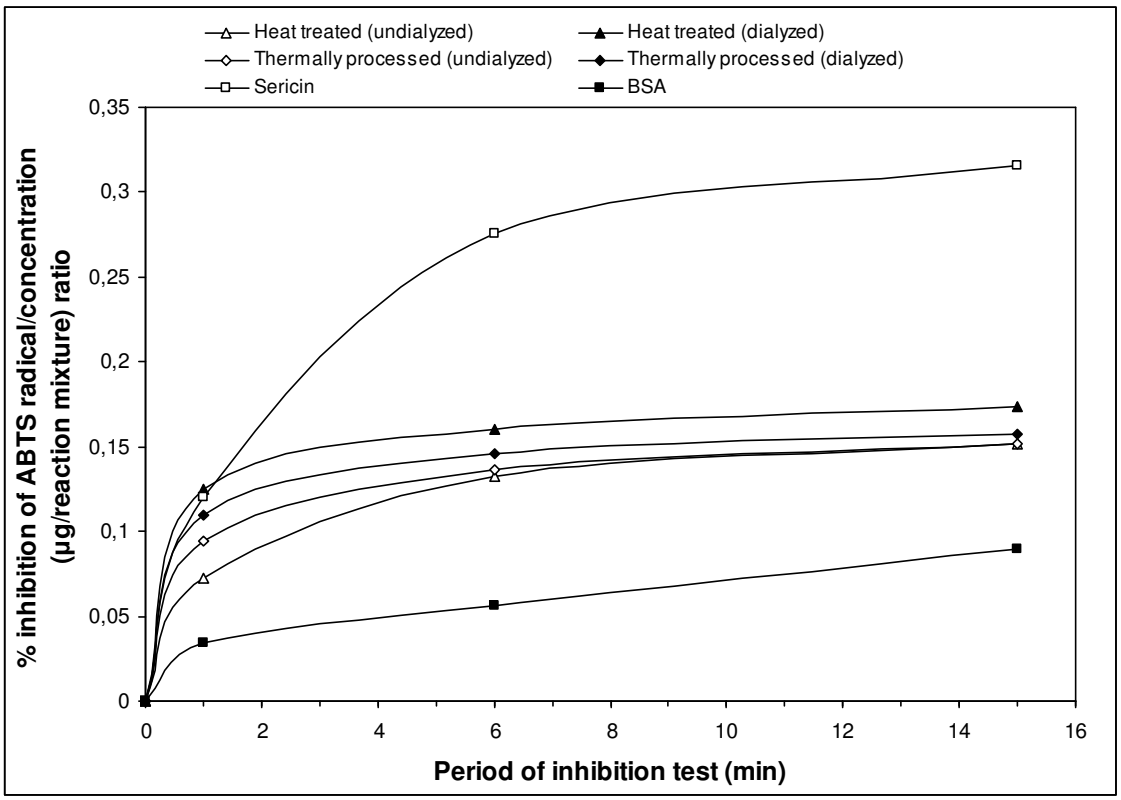


Figure 6.3. Antioxidant capacity of different lyophilized crude protein preparations of chick-peas against ABTS radical

In heat treated or thermally processed chick-peas, the dialysis increased the antioxidant activity of the lyophilized preparations. Thus, it seems that the heat treatment or thermal processing helped the separation of the prooxidants or antioxidant activity masking substances from these protein preparations by the dialysis. The dialysis increased also the antioxidant capacity of lyophilized crude protein preparations obtained from heat treated kidney-beans. However, in thermally processed kidney-beans dialysis reduced the antioxidant activity of lyophilized protein preparations. This result showed the varying response of heating on molecular properties of antioxidant proteins or prooxidants or antioxidant activity masking substances in kidney beans.

Table 6.4. Antioxidant capacities (AUC values) and inhibition/concentration ratios of different lyophilized crude protein preparations against ABTS free radical

Sample	% inhibition of ABTS radical/concentration (μg/reaction mixture) ratio		
	1 min	6 min	15 min
Sericin [AUC= 0.148 μmol Trolox/mg protein]			
	0.120 (0-600)[a]	0.276 (0-300)	0.315 (0-300)
BSA [AUC= 0.036 μmol Trolox/mg protein]			
	0.034 (0-1500)	0.056 (0-900)	0.089 (0-900)
Heat treated chick-peas (undialyzed) [AUC= 0.073 μmol Trolox/mg lyophilized protein preparation]			
	0.072 (0-900)	0.133 (0-600)	0.151 (0-600)
Heat treated chick-peas (dialyzed) [AUC= 0.091 μmol Trolox/mg lyophilized protein preparation]			
	0.125 (0-600)	0.160 (0-600)	0.173 (0-600)
Thermally processed chick-peas (undialyzed) [AUC= 0.077 μmol Trolox/mg lyophilized protein preparation]			
	0.095 (0-600)	0.136 (0-600)	0.152 (0-600)
Thermally processed chick-peas (dialyzed) [AUC= 0.082 μmol Trolox/mg lyophilized protein preparation]			
	0.110 (0-600)	0.146 (0-600)	0.157 (0-600)
Heat treated kidney -beans (undialyzed) [AUC= 0.056 μmol Trolox/mg lyophilized protein preparation]			
	0.068 (0-900)	0.093 (0-900)	0.124 (0-600)
Heat treated kidney-beans (dialyzed) [AUC= 0.067 μmol Trolox/mg lyophilized protein preparation]			
	0.068 (0-900)	0.121 (0-600)	0.137 (0-600)
Thermally processed kidney-beans (undialyzed) [AUC= 0.046 μmol Trolox/mg lyophilized protein preparation]			
	0.046 (0-1500)	0.083 (0-900)	0.094 (0-900)
Thermally processed kidney-beans (dialyzed) [AUC= 0.039 μmol Trolox/mg lyophilized protein preparation]			
	0.045 (0-1500)	0.063 (0-1500)	0.087 (0-900)

[a]The numbers in the parenthesis indicate the range of data used in calculations (μg/reaction mixture)

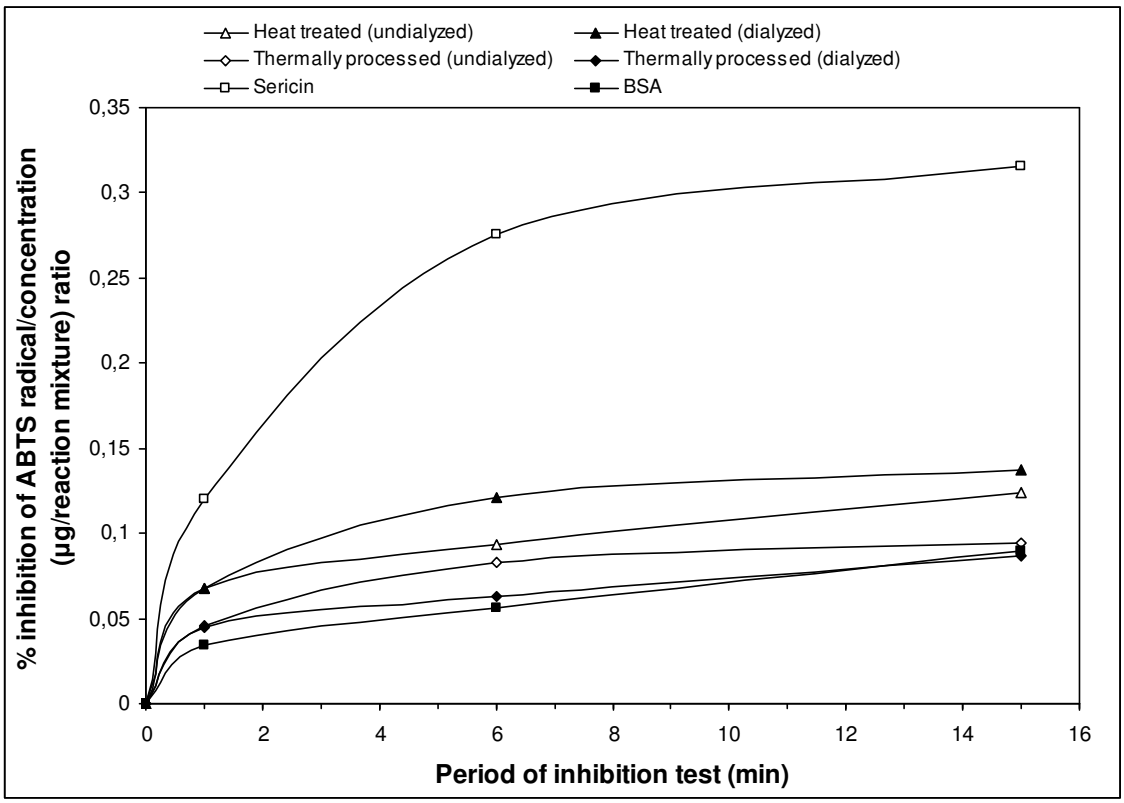


Figure 6.4. Antioxidant capacity of different lyophilized crude protein preparations of kidney-beans against ABTS radical

6.1.3. Fe^{+2} Chelating Capacity of Lyophilized Crude Protein Extracts of Heat Treated or Thermally Processed Chick-peas and Kidney-beans

Due to their prooxidant activity, the ability of a compound to bind metal atoms is also considered as a type of antioxidant activity (Synergistic antioxidant activity). As given in Table 6.5, sericin did not have a considerable Fe^{+2} chelating activity, whereas BSA showed its Fe^{+2} chelating activity only at high concentrations (Figure 6.5). The dialyzed and then lyophilized crude protein extracts obtained from heat treated kidney-beans and chick-peas showed moderate iron chelating activity. Thermal processing increased the chelating capacity of lyophilized chick-pea preparations by 80 %, while it reduced the chelating capacity of lyophilized kidney-bean preparations by 60 %.

Table 6.5. Fe^{+2} chelating capacity of lyophilized crude protein preparations of heat treated or thermally processed chick-peas and kidney-beans

Sample	EDTA equivalent of Fe^{+2} chelating capacity (μmol EDTA/ mg lyophilized protein preparation)
Sericin	0.003 (0-3000)[a]
BSA	0.03 (0-3000)
Heat treated chick-peas (dialyzed)	0.05 (0-1500)
Thermally processed chick-peas (dialyzed)	0.09 (0-600)
Heat treated kidney-beans (dialyzed)	0.07 (0-600)
Thermally processed kidney-beans (dialyzed)	0.03 (0-1500)

[a]The numbers in the parenthesis indicate the range of data used in calculations (μg/reaction mixture)

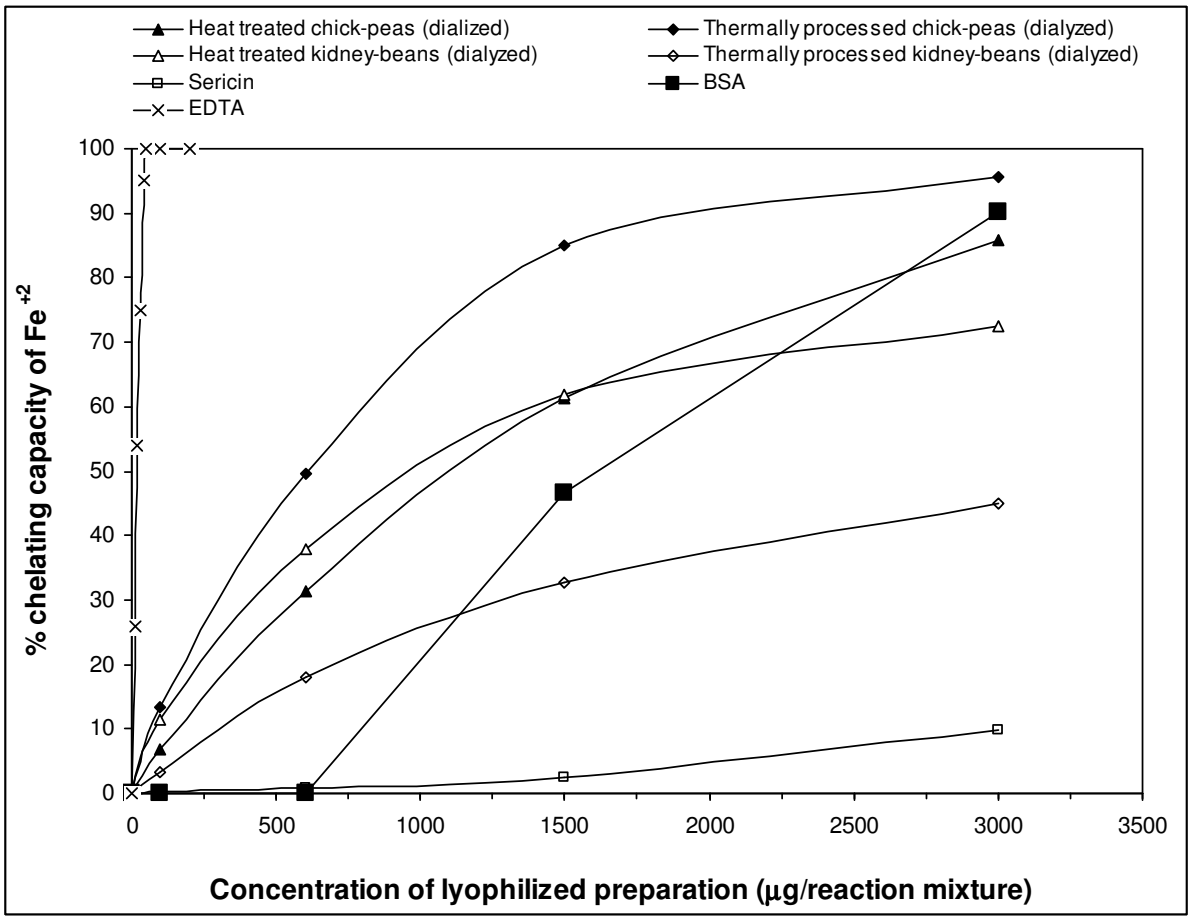


Figure 6.5. Fe^{+2} chelating capacity of different lyophilized crude protein preparations of heat treated or thermally processed chick-peas and kidney-beans

6.1.4. Application of Ammonium Sulfate Precipitation and Dialysis for Partial Purification of Crude Protein Extracts of Thermally Processed Chick-peas and Kidney-beans

In this work, the higher protein content and antioxidant activity of lyophilized chick-pea preparations indicate that this legume may be a suitable source for the extraction of antioxidant proteins. However, the lyophilized crude preparations may contain some impurities that may affect the antioxidant activity. Thus, in this study crude protein extracts were also partially purified by ammonium sulfate precipitation and dialysis. As seen in Tables 6.6 and 6.7, 90 % ammonium sulfate precipitation reduced the recovery of antioxidant activity of both chick-pea and kidney-bean extracts by 60 %. It seems that the ammonium sulfate did not precipitate some of the antioxidants at the studied saturation. The protein recovery of ammonium sulfate precipitation was also 54 % and 50 % for chick-pea and kidney-bean proteins, respectively. In chick-pea extracts, the application of dialysis reduced the specific antioxidant activity, recoveries of antioxidant activity and protein contend slightly. On the other hand, a slight increase was observed in the specific antioxidant activity of kidney-bean extracts, possibly due to removal of proteins lacking antioxidant activity by the dialysis.

6.1.4.1. Protein Content of Lyophilized Partially Purified Protein Extracts Obtained from Thermally Processed Chick-peas and Kidney-beans

Following dialysis, the ammonium sulfate precipitated extracts were lyophilized and assayed for protein content. By ammonium sulfate precipitation, an increase in the protein content of lyophilized preparation is expected. However, the results of protein assays showed the presence of 0.23 and 0.49 mg protein per mg of lyophilizates obtained from kidney-beans and chick-peas, respectively. These protein contents are very close to the protein contents of dialyzed lyophilized crude preparations (see Table 6.1). Thus, it is clear that besides proteins, other major hydrocolloids such as starch and pectin were also precipitated with the proteins. In fact, this result is not surprising since, it is well known from the fruit juice clarification that the removal of one of the

hydrocolloids in a colloidal system may cause also the precipitation of others (Cemeroğlu and Karadeniz, 2001). The abundance of starch in legumes and the high affinity of this carbohydrate to proteins also support this hypothesis (Baldwin, 2001).

6.1.4.2. Antioxidant Activity of Lyophilized Partially Purified Protein Extracts Obtained from Thermally Processed Chick-peas and Kidney-beans against ABTS Radical

The results of inhibition studies were given in Table 6.8. As seen in Figure 6.6., the antioxidant activity of ammonium sulfate precipitated, dialyzed and then lyophilized thermally processed chick-pea preparation is higher than that of kidney-bean preparation obtained by the same method. From the AUC values given in Table 6.9, the difference between the antioxidant capacities of two protein preparation was found 2.1 fold. This result is in line with the antioxidant activity measurements conducted in crude dialyzed and undialyzed preparations obtained from the thermally processed chick-peas and kidney-beans. However, it should be noted that the higher antioxidant activity of

Table 6.6. Ammonium sulfate precipitation of proteins from thermally processed chick-peas

Step	Vol. (mL)	Total antioxidant activity (µmol Trolox)[a]	Total protein (mg)	Specific antioxidant activity (µmol Trolox/mg)	Recovery of antioxidant activity (%)	Purification fold of antioxidant activity	Recovery of protein (%)
Crude extract							
	52.5	89.5	225	0.40	100	1	100
0-90 % ammonium sulfate precipitation							
	17.5	34.6	122	0.28	39	0.72	54
36h dialysis at 4 ^{o}C							
	21.0	26.9	113	0.24	30	0.60	50

[a] for a 15 min test period, 1µmol trolox equals to 0.15 µmol tyrosine

Table 6.7. Ammonium sulfate precipitation of proteins from thermally processed kidney-beans

Step	Vol. (mL)	Total antioxidant activity (µmol Trolox)[a]	Total protein (mg)	Specific antioxidant activity (µmol Trolox/mg)	Recovery of antioxidant activity (%)	Purification fold of antioxidant activity	Recovery of protein (%)
Crude extract							
	47	65.8	152	0.43	100	1	100
0-90 % ammonium sulfate precipitation							
	26	27.8	76	0.37	42	0.84	50
36h dialysis at 4 °C							
	30	19.8	49	0.40	30	0.93	32

[a] for a 15 min test period, 1µmol trolox equals to 0.15 µmol tyrosine

chick-pea preparations is related with their high protein content but not with their greater specific antioxidant activity (see Tables 6.6. and 6.7). By considering the AUC values, the antioxidant capacity of lyophilized preparations obtained by ammonium sulfate precipitation and dialysis were also compared with the antioxidant capacities of sericin and BSA. The chick-pea and kidney-bean preparations showed almost 2.8 and 1.3 fold higher antioxidant capacity than BSA, respectively. However, both preparations antioxidant activity was lower than that for the sericin. On the other hand, it should also be emphasized that compared to the antioxidant capacity of its crude lyophilized preparations (dialyzed or undialyzed), the antioxidant capacity of ammonium sulfate precipitated, dialyzed and then lyophilized chick-pea preparation is 1.2-1.3 fold higher.

Table 6.8. Inhibition of ABTS radical by ammonium sulfate precipitated lyophilized protein preparations of thermally processed Chick-peas and Kidney-beans

Sample	Concentration (µg/reaction mixture)	% inhibition of ABTS radical		
		1 min	6 min	15 min
Thermally processed chick-peas				
	100	18.9	28.9	32.6
	300	42.8	62.9	71.6
	600	63.5	83.6	89.0
	900	73.1	89.0	90.0
	1500	74.1	90.0	90.0
	3000	87.2	91.2	90.0
Thermally processed kidney-beans				
	100	14.7	20.7	23.4
	300	34.3	52.3	59.9
	600	52.1	75.7	84.2
	900	61.2	83.3	89.2
	1500	69.8	88.7	92.3
	3000	80.2	92.9	92.6

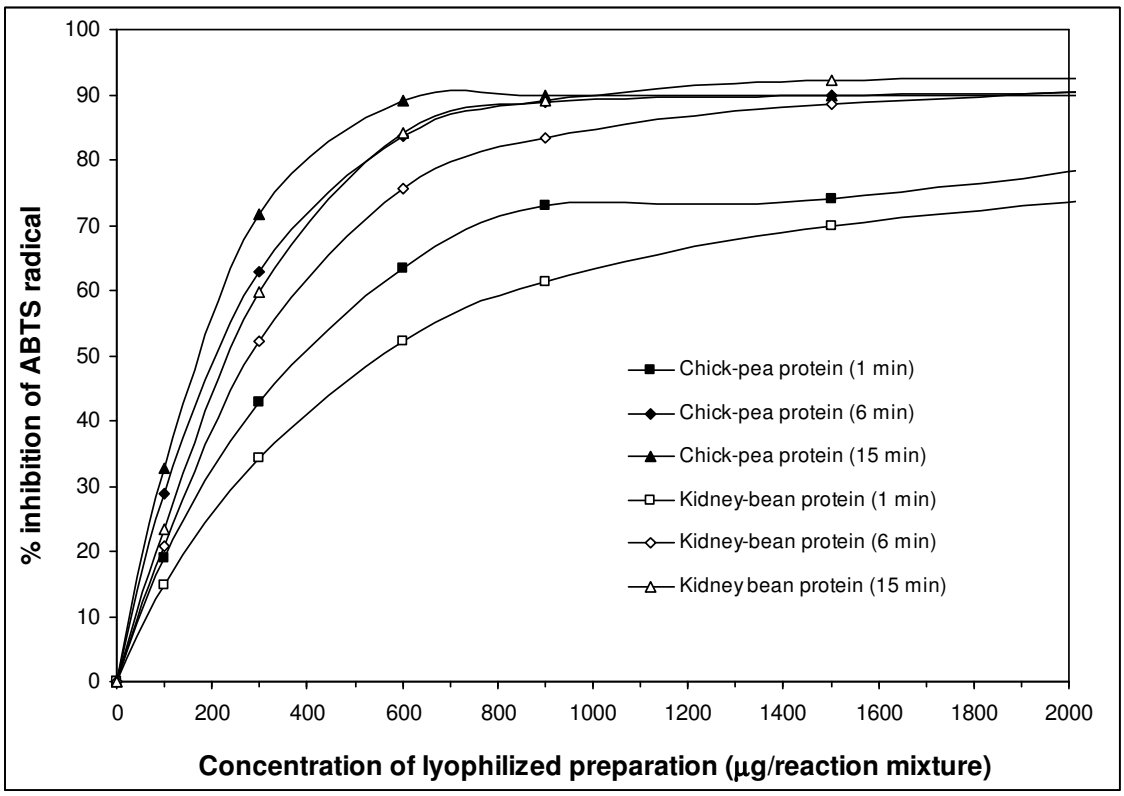


Figure 6.6. Inhibition of ABTS radical by ammonium sulfate precipitated lyophilized protein preparations of thermally processed chick-peas and kidney-beans

Table 6.9. Antioxidant capacities of ammonium sulfate precipitated lyophilized protein preparations of thermally processed chick-peas and kidney-beans

Source of protein	**% inhibition of ABTS radical/concentration (µg/reaction mixture) ratio**		
	1 min	**6 min**	**15 min**
Sericin [AUC= 0.148 µmol Trolox/mg protein]			
	0.120 (0-600)[a]	0.276 (0-300)	0.315 (0-300)
BSA [AUC= 0.036 µmol Trolox/mg protein]			
	0.034 (0-1500)	0.056 (0-900)	0.089 (0-900)
Thermally processed chick-peas [AUC= 0.102 µmol Trolox/mg lyophilized protein preparation]			
	0.115 (0-600)	0.156 (0-600)	0.247 (0-300)
Thermally processed kidney beans [AUC= 0.048 µmol Trolox/mg lyophilized protein preparation]			
	0.094 (0-600)	0.137 (0-600)	0.154 (0-600)

[a]The numbers in the parenthesis indicate the range of data used in calculations (µg/reaction mixture)

Since the partial purification did not increase the specific antioxidant activity and protein content of chick-pea preparations, as occurred during dialysis, the increased antioxidant activity by partial purification may be related with the removal of some prooxidants that reduce the antioxidant activity of crude preparations against ABTS radical or removal of antioxidant activity masking substances.

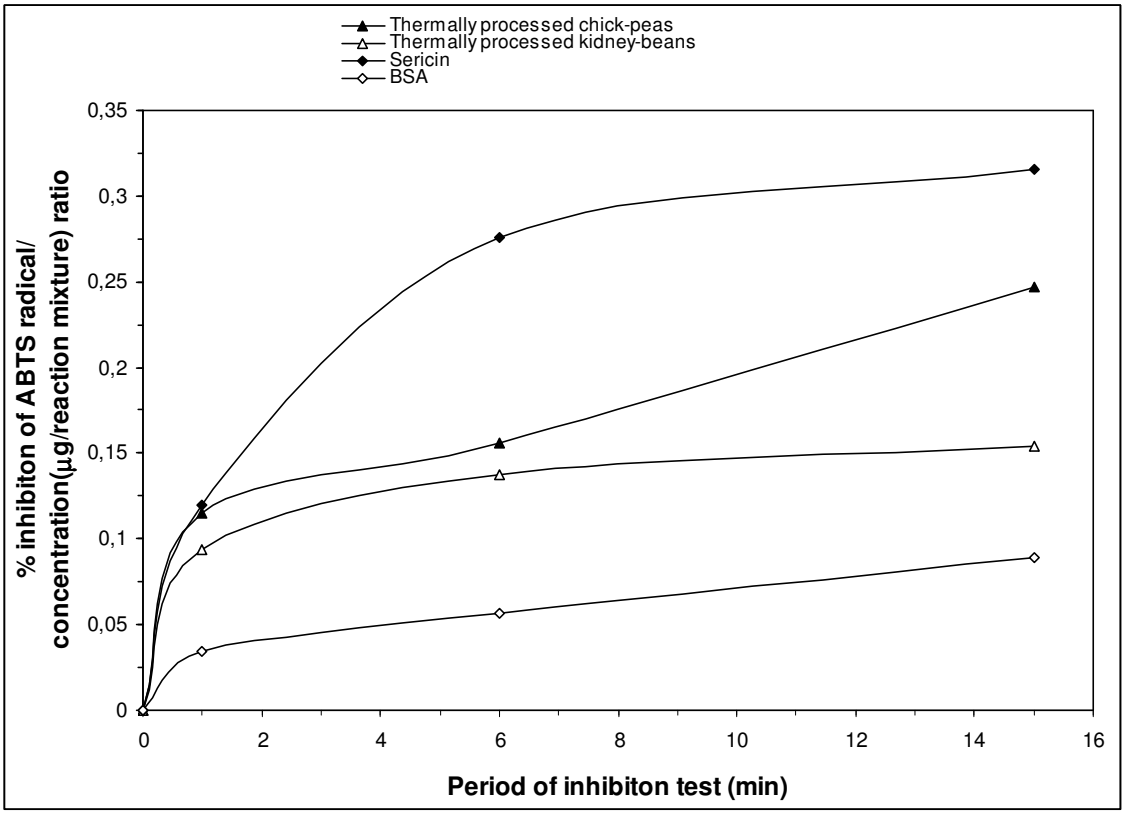


Figure 6.7. Antioxidant capacity of ammonium sulfate precipitated lyophilized thermally processed chick-pea and kidney-bean proteins against ABTS radical

6.1.5. Application of DEAE-cellulose Chromatography to Dialyzed Crude Protein Extracts for Partial Purification of Heat Treated or Thermally Processed Chick-pea Antioxidant Proteins

To increase the purity of antioxidant proteins, crude protein extracts from heat treated or thermally processed chick-peas were dialyzed and then applied to DEAE-cellulose fast flow columns. Our previous findings showed the relationship between antioxidant activity and protein content. As seen in Figure 6.8 and 6.9, the elution

profiles of antioxidant activities in DEAE-cellulose chromatograms of crude protein extracts always followed by protein peaks. Thus, this result confirms that the antioxidant activity measured is associated with proteins. In both chromatograms, only a little portion of protein was eluted from the columns by washing with the equilibration buffer. The antioxidant activities eluted from the columns by washing were also low and corresponded almost 10 and 20 % of the total antioxidant activity eluted from the chromatography of thermally processed or heat treated chick-pea proteins, respectively.

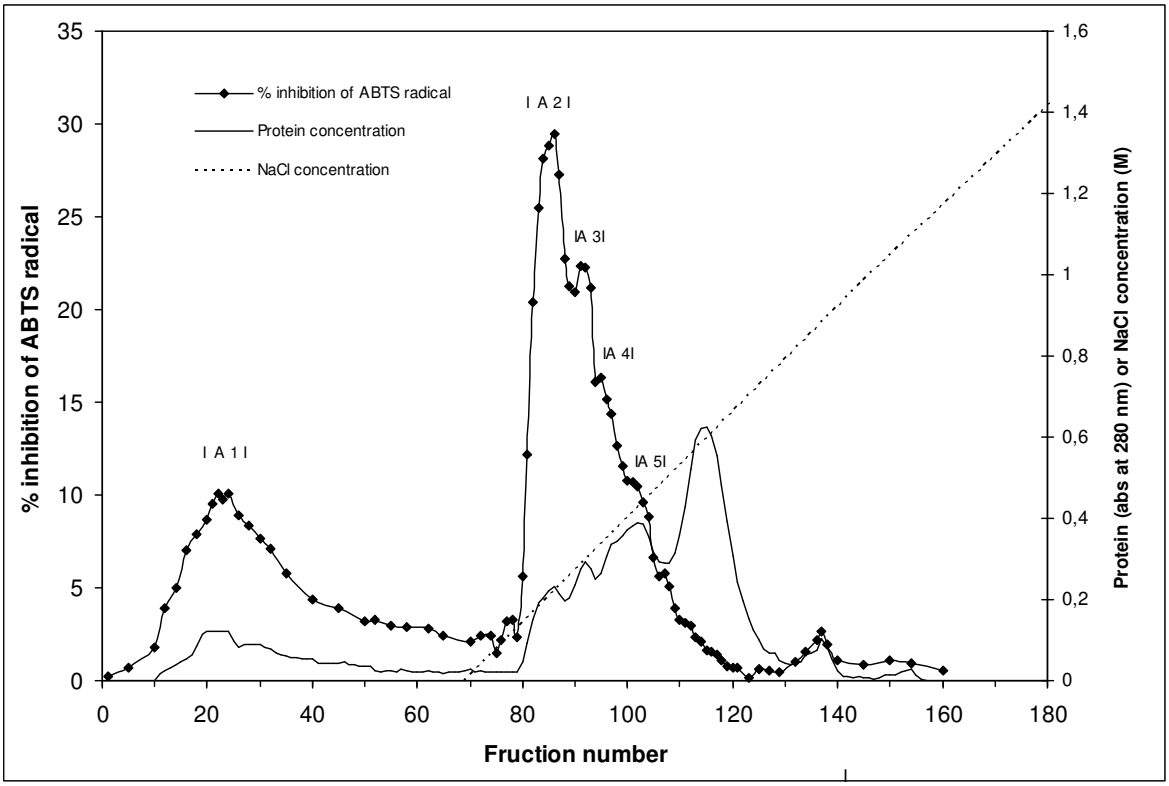


Figure 6.8. Purification of antioxidant proteins from heat treated chick-peas by DEAE-cellulose anion exchange chromatography (The crude extract was obtained by extraction of acetone powder at room temperature and a following dialysis; antioxidant activities were determined for 2 min inhibition test)

In both columns, the elution of the major antioxidant protein fractions started by the initiation of the linear gradient of NaCl and ended when NaCl concentration reached almost to 0.5 M. The protein peaks eluted above 0.5 M NaCl concentration did not show considerable antioxidant activity. Thus, it is clear that there is a specific group of protein responsible for the antioxidant activity. The chromatograms also showed the modification in proteins caused by thermal processing of chick-peas. As seen in Figure

6.9, the thermal processing increased mainly the antioxidant activity and protein content of first peak (B_2) came with the initiation of linear gradient. The thermal processing increased also the protein contents of other eluted fractions that show slight to moderate antioxidant activity. The increase in the antioxidant protein content and antioxidant activity in some fractions by thermal processing may be related with the modification of soluble proteins or solubilization of some membrane bound antioxidant proteins. In the literature, there are no studies related to the effect of thermal processing on antioxidant activity of chick-pea

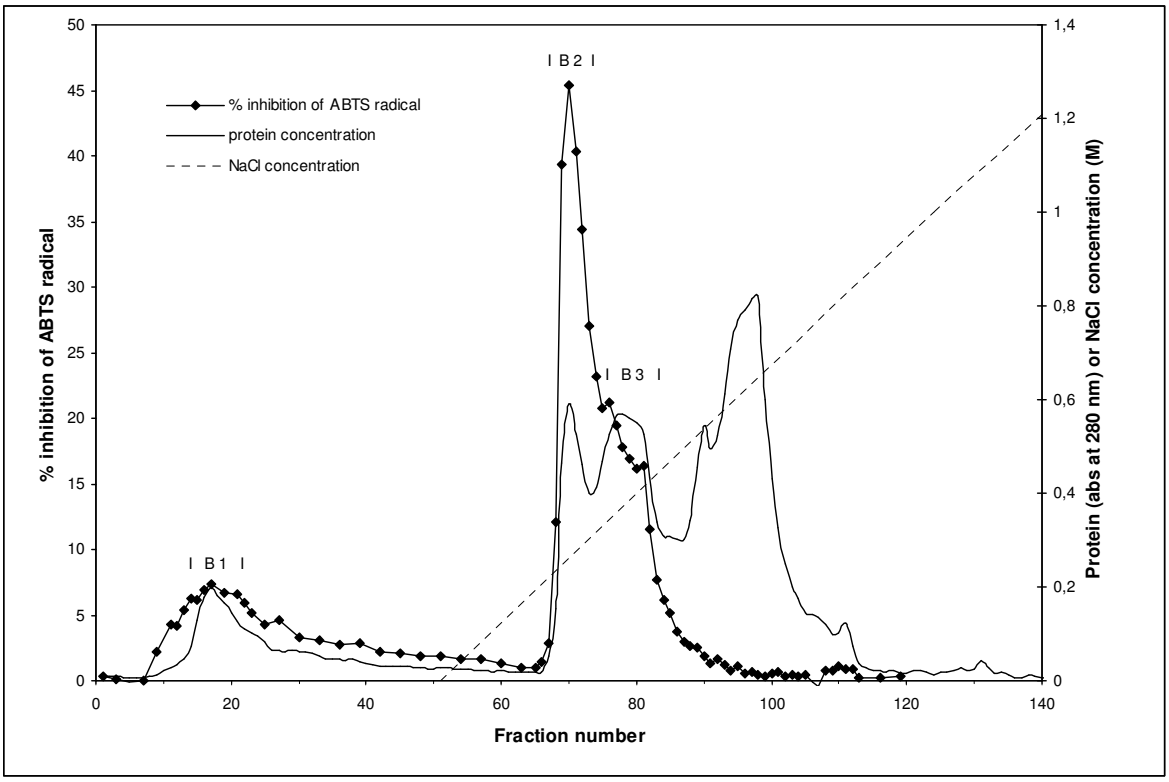


Figure 6.9. Purification of antioxidant proteins from thermally processed chick-peas by DEAE-cellulose anion exchange chromatography (The crude extract was obtained by extraction of acetone powder at room temperature and a following dialysis; antioxidant activities were determined for 2 min inhibition test)

proteins. However, it was reported that the thermal processing of bean albumins causes the aggregation of proteins by cross-links other than the disulfide bridges (Rocha et al, 2002).

Table 6.10. Purification of antioxidant proteins from dialyzed crude protein extracts of heat treated chick-peas[a]

Step	Vol. (mL)	Total antioxidant activity (µmol Trolox)[b]	Total protein (mg)	Specific antioxidant activity (µmol Trolox/mg)	Recovery of antioxidant activity (%)	Purification fold of antioxidant activity	Recovery of protein (%)
Crude extract							
	46	68.5	171.1	0.40	100	1	100
72h dialysis at 4°C							
	50	39.4	126.4	0.31	58	0.78	74
DEAE-cellulose anion exchange chromatography							
A_1	35	4.3	7.6	0.57	6.3	1.42	4.4
A_2	35	6.3	5.7	1.09	9.1	2.73	3.4
A_3	20	3.6	9.5	0.38	5.2	0.94	5.6
A_4	25	2.8	12.5	0.22	4.1	0.56	7.3
A_5	25	2.1	8.4	0.25	3.0	0.62	4.9

[a] extraction method: water extraction of acetone powder at room temperature; [b]for a 15 min test period 1µmol trolox equals to 0.15 µmol tyrosine

It is also interesting to note that the antioxidant activity eluted from DEAE-cellulose chromatography (Total: 35.5 µmol trolox) of thermally processed chick-peas was higher than the antioxidant activity loaded to this column (20.3 µmol trolox). The increase of antioxidant activity by purification was observed also in ammonium sulfate precipitation studies conducted with thermally processed chick-pea and kidney-bean proteins (see section 6.1.4.2). The increase in antioxidant activity was not observed following column chromatography of heat treated chick-pea proteins. Thus, this result once more showed the possible role of thermal processing in removal of some prooxidants or unmasking of the antioxidant activity of proteins after purification. Further studies are needed to better understand the actual reasons of the increased antioxidant activity of purified thermally processed proteins.

The purification parameters related to application of dialysis and DEAE-cellulose chromatographic procedures were also given in Table 6.10 and 6.11. As occurred in partial purification studies with ammonium sulfate, the dialysis reduced the specific antioxidant activity and recovery of antioxidant activity.

Table 6.11. Purification of antioxidant proteins from dialyzed crude protein extracts of thermally processed chick-peas[a]

Step	Vol. (mL)	Total antioxidant activity (µmol Trolox)[b]	Total protein (mg)	Specific antioxidant activity (µmol Trolox/mg)	Recovery of antioxidant activity (%)	Purification fold of antioxidant activity	Recovery of protein (%)
Crude extract							
	47	62.2	154.5	0.40	100	1	100
72h dialysis at 4°C							
	37.5	20.3	100.1	0.20	33	0.50	65
DEAE-cellulose anion exchange chromatography							
B_1	35	3.7	6.6	0.56	5.9	1.39	4.27
B_2	35	23.3	29.8	0.78	37.5	1.94	19.3
B_3	30	8.5	16.4	0.52	13.6	1.28	10.6

[a]extraction method: water extraction of acetone powder at room temperature; [b] for a 15 min test period 1µmol trolox equals to 0.15 µmol tyrosine

A decline in protein content was also observed by the dialysis. However, since our previous results indicated that the nonprotein substances are removed more effectively than the proteins, dialysis still serves to increase the protein/nonprotein materials ratio and antioxidant activity per mg of lyophilized preparation. By the application of DEAE-cellulose chromatography, the purity of some fractions increased moderately (1.3-2.7 fold) whereas some other proteins' specific antioxidant activity remained almost same or reduced below the specific antioxidant activity of dialyzed crude extract. For heat treated chick-peas, A_1 and A_2 fractions, and for thermally processed chick-peas B_1, B_2 and B_3 fractions are the fractions which showed an increase in specific antioxidant activity by the DEAE-cellulose chromatography.

6.1.5.1. Antioxidant Activity of DEAE-cellulose Chromatography Purified and Lyophilized Major Antioxidant Protein Fractions of Heat Treated or Thermally Processed Chick-peas

The main antioxidant protein fractions, A_2 from heat treated and B_2 from thermally processed chick-peas, were collected, dialyzed (24h), lyophilized and then tested for antioxidant activity (Table 6.12 and Figure 6.10). The antioxidant capacity of lyophilized A_2 fraction is almost same with that of sericin protein, but 3.8 fold higher than that of BSA protein (Table 6.13 and Figure 6.11). The antioxidant activity of B_2 fraction, on the other hand, was almost 0.7 fold lower than that of sericin, but 2.7 fold higher than that of BSA. These results indicate the greater antioxidant activity of lyophilized A_2 fraction than the lyophilized B_2 fraction. This apparently occurred as result of higher specific antioxidant activity of A_2 fraction. However, since the amount of lyophilized sample for B_2 is almost 3 fold higher than the amount of lyophilized sample for A_2, the total antioxidant activity of A_2 fraction is lower than that of B_2 fraction.

Table 6.12. Inhibition of ABTS radical by main antioxidant protein fractions purified from heat treated or thermally processed chick-peas by DEAE-cellulose column chromatography

Sample	Concentration (µg/reacton mixture)	% inhibition of ABTS radical		
		1 min	6 min	15 min
A_2 fraction of heat treated chick-peas				
	300	27.1	45.5	53.8
	600	36.7	65.3	77.5
	900	60.6	86.1	92.0
B_2 fraction of thermally processed chick-peas				
	300	16.2	21.1	21.7
	600	37.5	51.1	55.6
	900	59.4	77.6	80.6

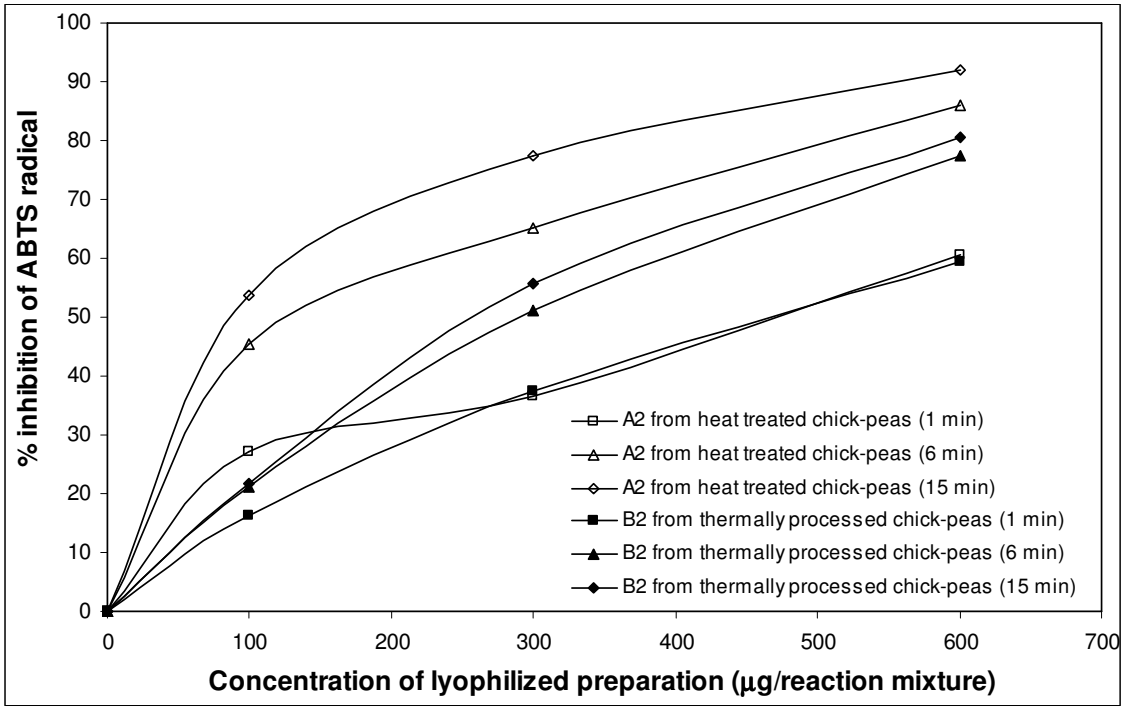


Figure 6.10. Inhibition of ABTS radical by main antioxidant protein fractions purified from heat treated or thermally processed chick-pea proteins by DEAE-cellulose chromatography

Table 6.13. Antioxidant capacities (AUC values) and inhibition/concentration ratios of main antioxidant protein fractions purified from heat treated or thermally processed chick-peas by DEAE-cellulose chromatography

Sample	% inhibition of ABTS radical/concentration (µg/reaction mixture) ratio		
	1 min	6 min	15 min
Sericin [AUC= 0.148 µmol Trolox/mg protein][a]			
	0.120 (0-600)[b]	0.276 (0-300)	0.315 (0-300)
BSA [AUC= 0.036 µmol Trolox/mg protein]			
	0.034 (0-1500)	0.056 (0-900)	0.089 (0-900)
A_2 fraction of heat treated chick-peas [AUC= 0.135 µmol Trolox/mg lyophilized protein]			
	0.137 (0-300)	0.241 (0-300)	0.287 (0-300)
B_2 fraction of thermally processed chick-peas [AUC= 0.098 µmol Trolox/mg lyophilized protein]			
	0.129 (0-300)	0.174 (0-300)	0.190 (0-300)

[a]The numbers in the parenthesis indicate the range of data used in calculations (µg/reaction mixture)

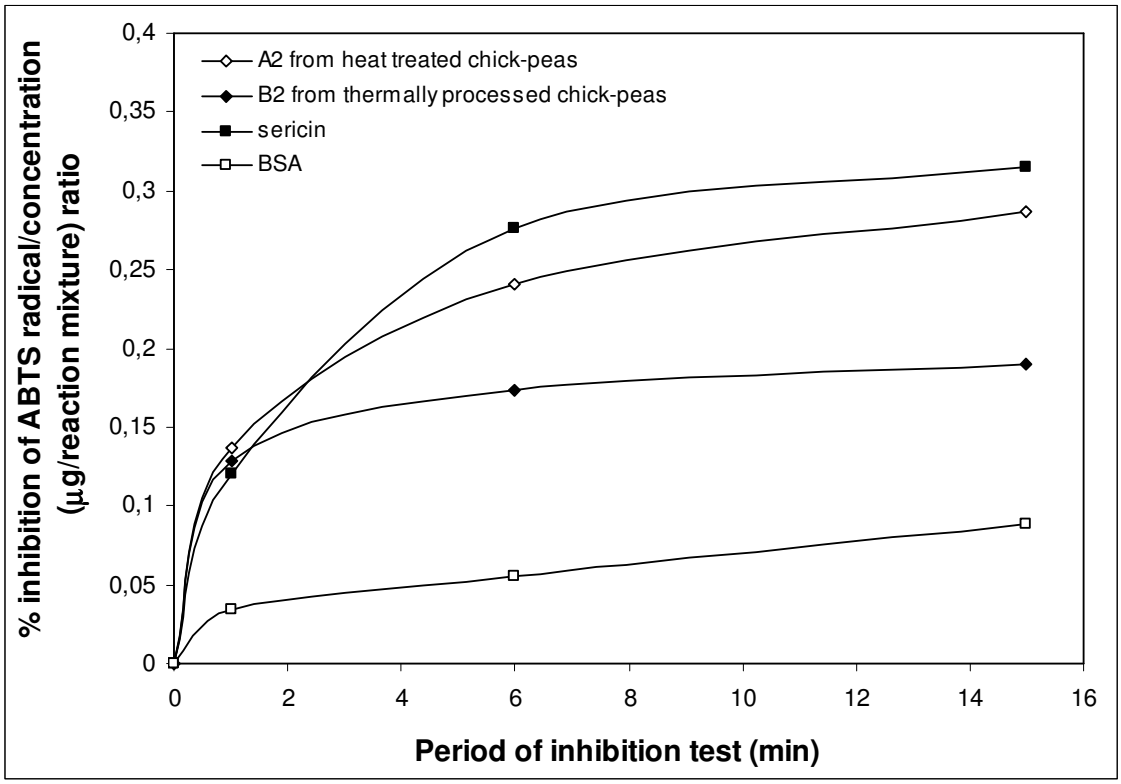


Figure 6.11. Antioxidant capacity of main antioxidant protein fractions (A2 and B2)purified from heat treated or thermally processed chick-pea proteins by DEAE-cellulose chromatography

6.2. Studies with Crude Protein Extracts of Chick-peas Obtained by Hot Acidic Hydrolysis or Hot Water Extraction Conducted at Different pH Values

The hot acidic hydrolysis and extraction at extreme pH values were applied mainly for the modification of the antioxidant activity of proteins and increase of the extraction yields of proteins, respectively. In this part of the study, the legumes were directly processed to acetone powder without any heat application and then the extraction of acetone powder was conducted at 85 ^{o}C (hot extraction). The high temperature used was selected to modify the antioxidant activity of proteins under extreme conditions and to inactivate the enzyme lipoxygenase (this was confirmed by activity measurements). Also, to better monitor the low molecular weight antioxidant proteins that may be extracted or formed under extreme conditions, PVPP was not used in extractions.

6.2.1. Antioxidant Activity and Protein Content of Crude Protein Extracts of Chick-peas Obtained by Hot Acidic Hydrolysis

In the literature the enzymatic hydrolysis with proteases has been mostly applied to obtain protein hydrolysates (Amarowicz and Shahidi, 1997, Rival et al, 2001, Chen et al, 1996). It is also well known that heating in highly acidic medium causes the hydrolysis of proteins (Bull and Hahn, 1948, Greenberg and Burk, 1927). Thus, in this study, limited acidic hydrolysis was applied to determine the effect of this treatment on antioxidant activity and protein content of crude protein extracts of chick-peas. As seen in Table 6.14, the application of 30 min heating in presence of 1.5 M HCl gave a crude extract with high specific antioxidant activity. The application of dialysis reduced the specific antioxidant activity of 30 min acid hydrolyzed samples moderately. Thus, the increased antioxidant activity of hydrolyzed sample is not mainly due to low molecular substances formed by acidic hydrolysis. The reduction of hot hydrolysis period to 10 min, on the other hand, reduced both the protein content and antioxidant activity, but increased the specific antioxidant activity slightly.

The results showed that the 30 min acidic hydrolysis at 85 ^{o}C may be used to increase the protein content and antioxidant activity of crude protein extracts. However, it should be noted that the application of hot acidic hydrolysis caused also the formation of Maillard reaction products. In hydrolyzed samples, the light brown color formed during hydrolysis indicated the presence of Maillard reaction products in crude extracts. The Maillard reaction products are formed by reaction between reducing sugars and amino acids or proteins (Yoshimura et al, 1997). The antioxidant activity of Maillard reaction products was reported by many different workers (Murakami et al, 2002, Yoshimura et al, 1997, Duh et al, 2001). However, the use of preparations containing Maillard reaction products needs some toxicological testing, since these products have mutagenic activity (Murakami et al, 2002).

Table 6.14. Antioxidant activity and protein contents of crude protein extracts of chick-peas obtained by hot acidic hydrolysis

Extraction conditions of acetone powder	Protein (mg/mL)	Antioxidant activity (µmol trolox/mL)	Specific antioxidant activity (µmol trolox/mg)
Suspension of acetone powder in 1.5 M HCl / continuous stirring at 85 °C for 30 min / neutralization / continuous stirring at room temperature for 30 min / centrifugation			
+ 24h incubation at 4 °C	4.19	2.34	0.56
+ 24h dialysis at 4 °C	2.95	1.26	0.43
Suspension of acetone powder in 1.5 M HCl / continuous stirring at 85 °C for 10 min / neutralization / continuous stirring at room temperature for 50 min / centrifugation			
+ 24h incubation at 4 °C	2.80	1.65	0.59
+ 24h dialysis at 4 °C	1.97	0.79	0.41

6.2.2. Antioxidant Activity and Protein Content of Crude Protein Extracts of Chick-peas Obtained by Hot Water Extraction at Different pH Values

The results of hot extractions conducted at different pH values were given in Table 6.15. The application of hot extraction at pH 6.5 and 85 °C for 60 min increased the specific antioxidant activity of extracts to almost 80 % of the specific antioxidant activity of 30 min acid hydrolyzed sample without a considerable color change in the protein extract. The application of dialysis, on the other hand, reduced the protein content and antioxidant activity almost 60 and 75 %, respectively. The reduction of heating period to 30 min at pH 6.5 and 85 °C reduced the specific antioxidant activity and protein content. Also, 30 % reduction occurred in antioxidant activity by reduction of heating period. Conducting hot extraction for 30 min at 85 °C by changing extraction pH from 6.5 to 9.5 did not increase the specific antioxidant activity. However, this increased the protein content of extracts almost 100 %, compared with the protein contents of samples extracted at pH 6.5. On the other hand, conducting extraction for 30 min at 85 °C by changing extraction pH to 2.5 increased the specific antioxidant activity to the highest level obtained in extraction studies. The protein content of the extract at pH 2.5 reduced considerably. However, it seems that the conditions at this pH were very

suitable for the selective extraction of antioxidant proteins and/or other potential antioxidants such as peptides, protein-phenolic or carbohydrate-phenolic associates. The dialysis of the extract obtained at pH 2.5 reduced the specific antioxidant activity almost 40 %. Thus, it is clear that the important part of antioxidants in this extract is low molecular weight compounds.

Table 6.15. Antioxidant activity and protein contents of crude protein extracts of chick-peas obtained by hot extraction at different pH values

Extraction conditions of acetone powder	**Protein (mg/mL)**	**Antioxidant activity (µmol trolox/mL)**	**Specific antioxidant activity (µmol trolox/mg)**
Suspension of acetone powder in deionized water (pH was almost 6.5) / continuous stirring at 85 ^{o}C for 60 min / centrifugation			
+ 24h incubation at 4 ^{o}C	3.70	1.70	0.46
+ 24h dialysis 4 ^{o}C	1.37	0.45	0.32
Suspension of acetone powder in deionized water (pH was almost 6.5) / Continuous stirring at 85 ^{o}C for 30 min + at room temperature for 30 min / centrifugation			
+ 24h incubation at 4 ^{o}C	3.20	1.17	0.37
Suspension of acetone powder in deionized water / adjustment of pH to 9.5 / continuous stirring at 85 ^{o}C for 30 min + at room temperature for 30 min / adjustment of pH to 6.5 / centrifugation			
+ 24h incubation at 4 ^{o}C	6.54	2.21	0.34
+ 24h dialysis at 4 ^{o}C	2.57	0.37	0.14
Suspension of acetone powder in deionized water / adjustment of pH to 2.5 / continuous stirring at 85 ^{o}C for 30 min + at room temperature for 30 min / adjustment of pH to 6.5 / centrifugation			
+ 24h incubation at 4 ^{o}C	1.83	1.23	0.67
+ 24h dialysis at 4 ^{o}C	1.39	0.56	0.41

6.2.3. Application of Ammonium Sulfate Precipitation and Dialysis for Partial Purification of Crude Protein Extracts of Chick-peas Obtained by Hot Extraction Conducted at pH Values Close to Neutrality

The summary of the ammonium sulfate precipitation of crude protein extracts obtained by 30 min extraction of chick-pea acetone powder at pH 6.5 and 85 ^{o}C was given in Table 6.16. For chick-pea crude proteins obtained by hot extraction, the recovery of protein for ammonium sulfate precipitation was almost 15-20 % higher than those of the previous ammonium sulfate precipitations conducted for crude proteins extracted from thermally processed chick-peas and kidney-beans at room temperature (see Table 6.6. and 6.7). However, similar to previous precipitations the recovery of antioxidant activity by ammonium sulfate precipitation was low. In hot extracted chick-pea crude proteins, the dialysis of ammonium sulfate precipitates further reduced the recoveries of antioxidant activity and protein. Thus, it seems that the removal of low molecular substances by dialysis and/or insolubilization during dialysis is very high in ammonium sulfate precipitated crude protein extracts obtained by hot water extraction.

Table 6.16. Ammonium sulfate precipitation of crude chick-pea proteins obtained by hot water extraction conducted at pH values close to neutrality

Step	Vol. (mL)	Total antioxidant activity (µmol Trolox)[a]	Total protein (mg)	Specific antioxidant activity (µmol Trolox/mg)	Recovery of antioxidant activity (%)	Purification fold of antioxidant activity	Recovery of protein (%)
Crude extract							
	100	145.9	353	0.41	100	1	100
0-90 % ammonium sulfate precipitation							
	28	53.6	240	0.22	37	0.54	68
36h dialysis at 4 ^{o}C							
	40	21.6	76	0.28	15	0.69	22

[a] for a 15 min test period, 1 µmol trolox equals to 0,15 µmol tyrosine

6.2.4. Application of DEAE-cellulose Chromatography for Partial Purification of Crude Chick-pea Antioxidant Proteins Obtained by Hot Extraction Conducted at pH Values Close to Neutrality

For purification of antioxidant proteins from crude protein extracts obtained by 30 min extraction of chick-pea acetone powder at pH 6.5 and 85 ^{o}C, a crude extract was applied to DEAE-cellulose column. To determine the antioxidant activity and protein profiles of both low and high molecular weight fractions, the crude extract was not dialyzed before chromatography (dialysis was applied before other chromatographic separations given in Figures 6.8 and 6.9).

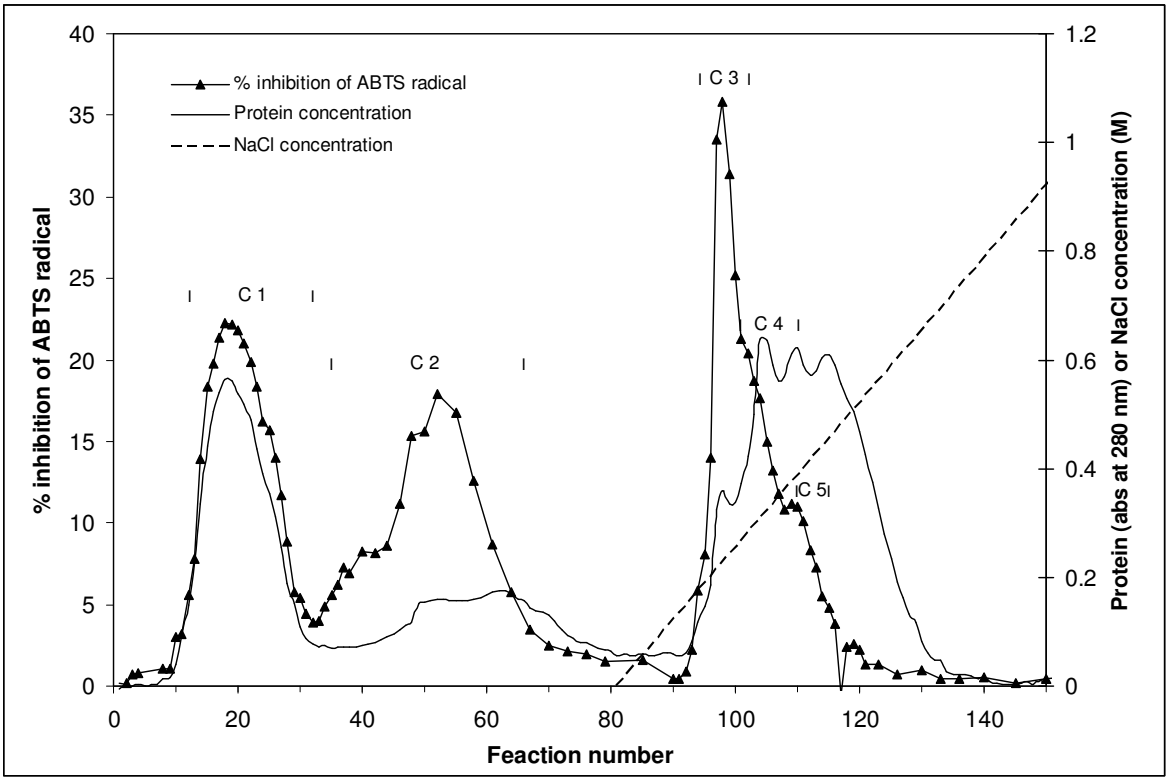


Figure 6.12. Purification of antioxidant proteins from chick-pea crude protein extracts by DEAE-cellulose anion exchange chromatography (The crude extract was obtained by 30 min extraction of acetone powder at pH 6.5 and 85 ^{o}C; antioxidant activities were determined for 2 min inhibition test)

As seen in Figure 6.12, for the DEAE-cellulose chromatography of crude protein extract, three main peaks, C_1, C_2 and C_3, were obtained for the antioxidant activity. From these peaks C_3 peak contained two shoulders (C_4 and C_5), confirmed by the

protein peaks following the antioxidant activity peaks. The elution of significant amount of antioxidant activity (followed by protein peaks) from the column by washing suggests the presence protein fractions which can not bound by the DEAE-cellulose anion exchange column. During previous DEAE-cellulose column chromatographic studies of dialyzed crude extracts of thermally processed or heat treated chick-peas, there was only little amounts of DEAE-cellulose unbound proteins (see section 6.1.5). The crude protein extract obtained by hot extraction was not dialyzed. Thus, it is possible that the unbound substances are low molecular weight proteins that contain almost no or little negatively charged groups that contribute to anion exchange reactions in DEAE-cellulose column. The rapid elution of C_1 suggests the lack of negative charges in these proteins whereas later elution of C_2 suggests slight binding due to limited number of negative charges. The lack of charges in these protein fractions may also be due to the complex formation of these proteins with neutral polysaccharides and masking of negative charges. Further studies are needed for full characterization of antioxidant proteins in chick-peas.

The partial purification parameters for the antioxidant activity of the eluted protein fractions were also given in Table 6.17. The results indicated that the highest purification

Table 6.17. Purification of antioxidant proteins from chick-peas[a]

Step	Vol. (mL)	Total antioxidant activity(µmol Trolox)[b]	Total protein (mg)	Specific antioxidant activity (µmol Trolox/mg)	Recovery of antioxidant activity (%)	Purification fold of antioxidant activity	Recovery of protein (%)
Crude extract							
	50	58.5	160	0.37	100	1.00	100
DEAE cellulose anion exchange chromatography							
C1	95	16.7	9.1	1.83	28.5	5.00	5.7
C2	150	16.8	6.1	2.76	28.8	7.53	3.8
C3	35	5.9	13.1	0.45	10.2	1.24	8.2
C4	40	5.6	22.4	0.25	9.6	0.68	14.0
C5	25	2.3	9,5	0.24	3.9	0.65	5.9

[a] extraction method: water extraction of acetone powder at 85° C, [b] for 15 min test period, 1 µmol trolox equals to 0.15 µmol tyrosine

folds were obtained for the unbound fractions, C_1 and C_2. It is clear that the lack of negative charges in these proteins capable to contribute anion exchange reactions was responsible for the separation and resulting purification of these fractions. A slight increase in purify of C_3 was also observed, whereas other fractions' purity declined due to small amount of antioxidant activity but high protein content in these fractions.

On the other hand, the elution profile (a main peak and two shoulders) of antioxidant activity by the initiation of NaCl gradient was quite similar with that of thermally processed chick-peas (see Figure 6.9). Thus, it seems that the hot extraction and thermal processing cause similar modifications in chick-pea proteins bind to DEAE-cellulose. However, in chromatography of chick-pea crude proteins obtained by hot extraction, the antioxidant activity loaded to column was higher than the antioxidant activity eluted. Thus, it is clear that, unlike to thermal processing, hot extraction was not effective in removal of prooxidants or antioxidant activity masking substances.

CHAPTER 7

CONCLUSIONS

- Kidney-beans and chick-peas contain antioxidant proteins with free radical scavenging and metal chelating capacity.

- Lyophilized crude proteins from chick-peas contain more protein and higher free radical scavenging activity than lyophilized crude proteins from kidney-beans.

- Free radical scavenging activity of lyophilized crude proteins from heat treated or thermally processed kidney-beans and chick-peas is higher than the free radical scavenging activity of bovine serum albumin. However, all lyophilized crude proteins showed lower free radical scavenging activity than sericin.

- Lyophilized crude proteins from chick-peas and kidney-beans show greater iron chelating capacity than bovine serum albumin and sericin.

- Thermal processing does not cause a significant change in free radical scavenging capacity of lyophilized crude chick-pea proteins. However, it increases their iron chelating capacity. In contrast, thermal processing reduces both free radical scavenging and iron chelating capacity of lyophilized crude kidney-bean proteins.

- Dialysis increases the free radical scavenging capacity of lyophilized crude thermally processed or heat treated chick-pea and heat treated kidney-bean proteins. However, it does not cause a considerable change in the free radical scavenging capacity of thermally processed kidney-bean proteins.

- Partial purification by ammonium sulfate precipitation or DEAE-cellulose anion exchange chromatography removes the substances that mask the antioxidant activity or prooxidants from crude protein extracts of thermally processed chick-peas and this causes an increase in the free radical scavenging activity of these extracts.

- DEAE-cellulose chromatography showed the presence of five and three antioxidant protein fractions in heat-treated and thermally processed chick-peas respectively. The free radical scavenging activity of one of the purified antioxidant protein fractions from heat treated chick-peas was very close to that of sericin.

- Hot acidic hydrolysis may be used to increase the protein content and specific antioxidant activity of crude protein extracts. However, it causes also the formation of undesirable light brown colored Maillard reaction products.

- Hot extraction at pH 2.5 can be used for the selective extraction of antioxidant proteins, but this method is not very effective for the extraction of other proteins.

- Hot extraction at pH 9.5 is the most effective method for the extraction of antioxidant proteins and other proteins.

FUTURE EXPECTATIONS

- The results of this study clearly showed the free radical scavenging activity and iron chelating capacity of chick-pea proteins. However, further studies are needed to test the antioxidant effects of these proteins in real food systems.

- The presence of antioxidant protein fractions in legumes shows the existence of genes responsible for the synthesis of these proteins. Molecular biology may be used as a tool to obtain legumes with high antioxidant protein content. A detailed scavenging in different legumes cultivars is needed.

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APPENDIX A

Tyrosine Standard for ABTS Radical Cation Discoloration Assay

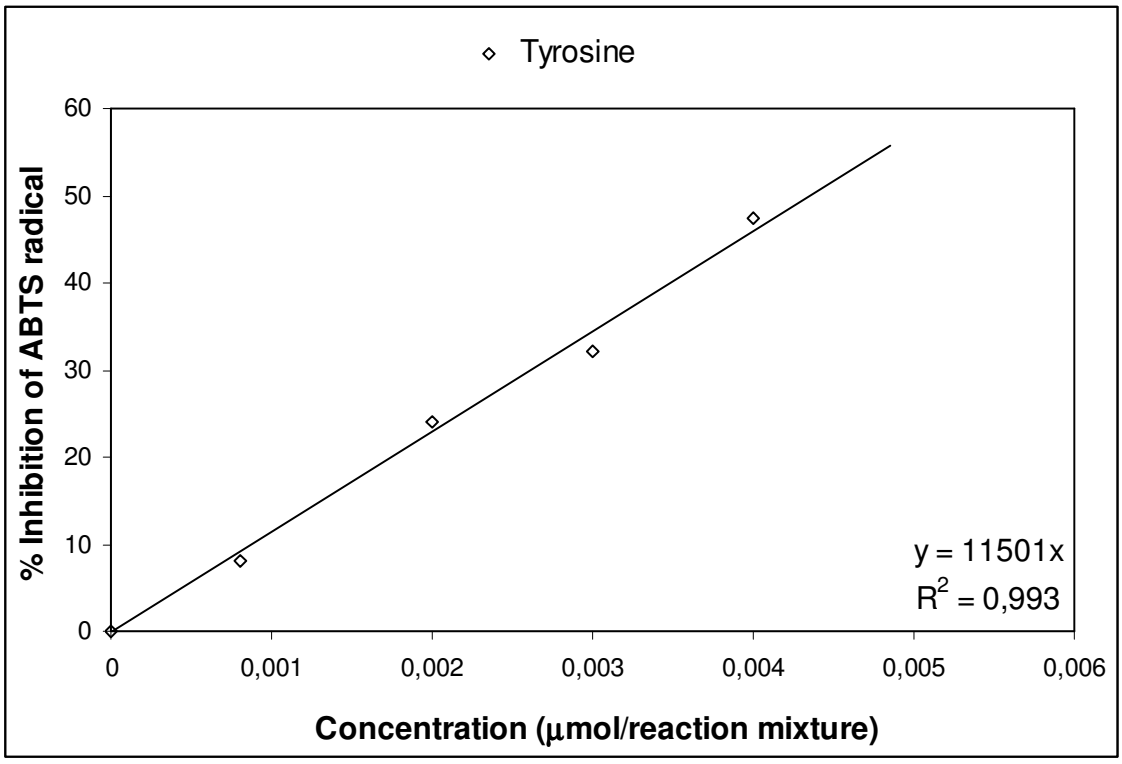

Figure A.1. Standard curve for Tyrosine

APPENDIX B

Trolox Standard for ABTS Radical Cation Discoloration Assay

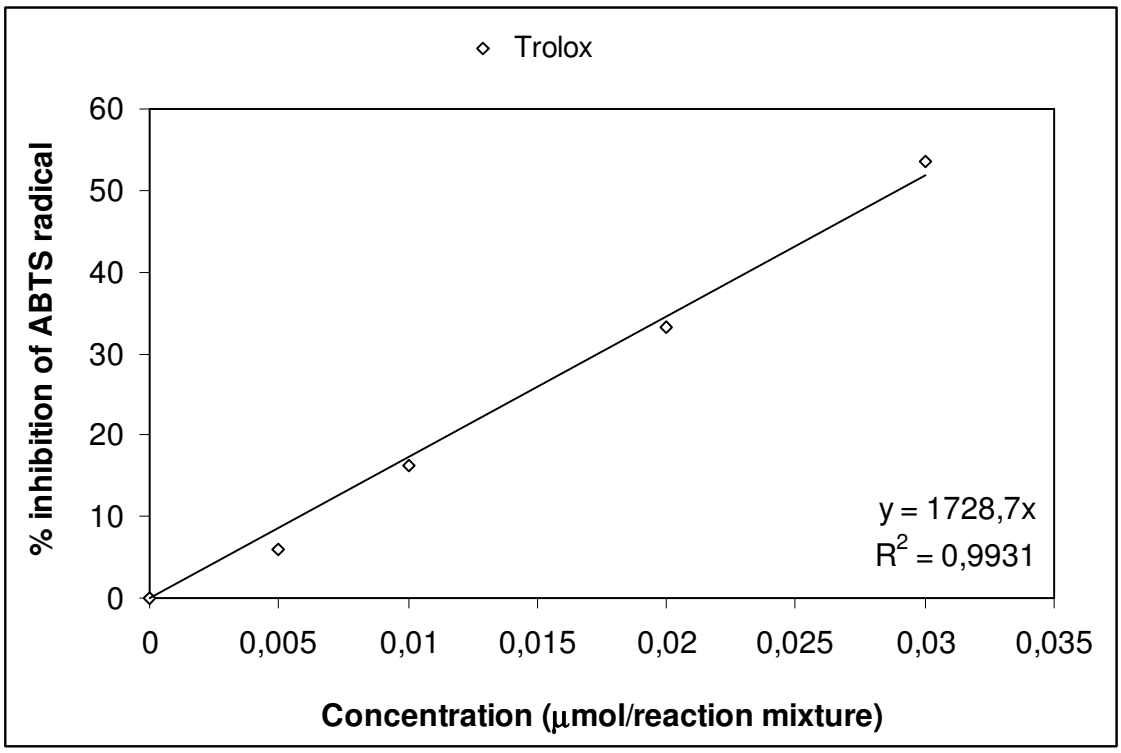

Figure B.1. Standard curve for Trolox

APPENDIX C

EDTA Standard for Fe^{+2} chelating capacity assay

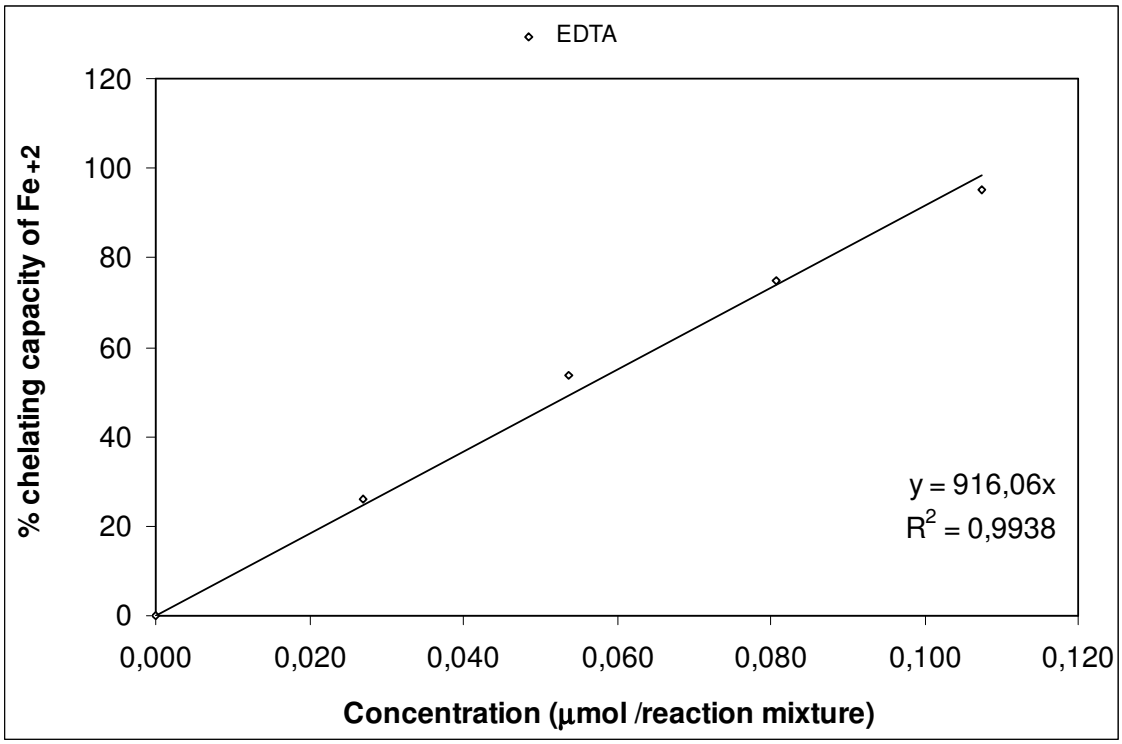

Figure C.1. EDTA Standard for Fe^{+2} Chelating capacity assay

APPENDIX D

BSA Standard for Lowry Method

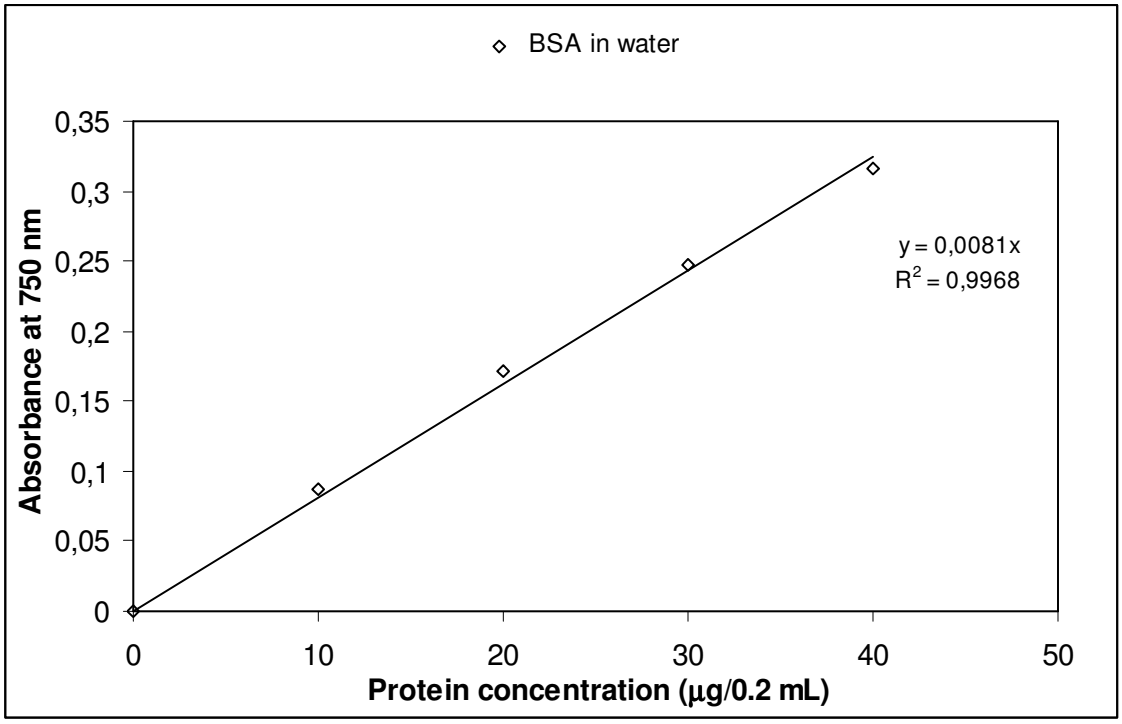

Figure D.1. Protein standard curve for Lowry method